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Hyperforin induced activation of the Pregnane X Receptor is influenced by the Organic Anion Transporting Polypeptide 2B1 (OATP2B1)

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List of nonstandard abbreviations: BSP, bromosulfophthalein; CCF, competitive counterflow; DMEM, Dulbecco's modified Eagles medium; E₁S, estrone 3-sulfate; FCS, fetal calve serum; HANKs, Hanks balanced salt solution; MDCKII cells, Madin-darby canine kidney epithelial cells; OATP, Organic Anion Transporting Polypeptide; PBS, phosphate buffered saline; PXR, Pregnane X Receptor; SJW, St. John's wort.

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Abstract

The herbal remedy St. John's wort (SJW) is used in the treatment of mild depressive symptoms and is known for its drug-drug interaction potential, where enhanced expression of CYP3A4 modifies clearance of concomitantly applied substrate drugs. Hyperforin is one constituent of SJW, which alters CYP3A4 expression by activation of the nuclear receptor Pregnane X Receptor (PXR). However, little is known about the transmembrane transport of hyperforin. One membrane protein, which modulates cellular entry of drugs is the organic anion transporting polypeptide (OATP) 2B1. It was aim of this study to test whether hyperforin interacts with this transport protein. Transport inhibition studies and competitive counterflow experiments suggested that hyperforin is a substrate of OATP2B1. This notion was validated showing that presence of OATP2B1 enhanced the hyperforin-induced PXR activation in cell based luciferase assays. Moreover, in Caco-2 cells transcellular transport of the known OATP2B1 substrate atorvastatin was changed in the presence of hyperforin, resulting in an increased efflux-ratio. Eleven commercially available SJW formulations were assessed for their influence on OATP2B1-mediated transport of estrone 3-sulfate and for their impact on CYP3A4 promoter transactivation. The correlation between effect size and the hyperforin content as determined by HPLC-UV suggested that hyperforin is the major determinant. Our results indicate an interaction between hyperforin and OATP2B1, which is not only known to contribute to hepatocellular uptake, but also intestinal absorption of its substrates. These findings extend the complexity of mechanisms that should be considered, when evaluating the interaction potential of SJW preparations.

Key words: transmembrane transport, OATP2B1, St. John's wort, Pregnane X receptor, hyperforin

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Introduction

St. John's wort is an herbal extract of *Hypericum perforatum* and is commonly taken to treat depressive symptoms. The extract is a complex mixture of structurally diverse constituents including flavonol glycosides, phloroglucinols, proanthocyanidins, naphthodianthrones, and phenylpropanoids (Nahrstedt and Butterweck, 1997). The naphthodianthrones pseudohypericin and hypericin were originally assumed to be the active components of *Hypericum* extracts (Suzuki et al., 1984). Accordingly, hypericin became the constituent on which therapeutically used extracts are standardized. However, even if still not fully understood more recent studies suggest that anti-depressive activity is most likely linked to the phloroglucinol hyperforin. (Mennini and Gobbi, 2004).

Beside its antidepressant activity, St. John's wort is known for its pronounced influence on expression and activity of genes involved in drug metabolism (Soleymani et al., 2017). Indeed, hyperforin enhances expression and activity of CYP3A4, whereby modifying the first-pass metabolism and clearance of concomitantly applied substrates (Wang et al., 2013). Similar results were shown for the efflux transporter P-glycoprotein (P-gp, ABCB1, MDR1), where treatment with St. John's wort extracts increased intestinal ABCB1 expression, whereby explaining the reduced bioavailability of ABCB1 substrates (Durr et al., 2000). The underlying mechanism is the activation of the Pregnane X Receptor (PXR) (Kliwer et al., 2002). PXR is a nuclear receptor functioning as ligand activated transcription factor of a gene network summarizing various proteins involved in drug metabolism including CYP3A4 (Lehmann et al., 1998), the efflux transporter P-gp (Geick et al., 2001), the multidrug-resistance protein 3 (ABCC3, MRP3) (Aleksunes and Klaassen, 2012), the organic anion transporting polypeptide 1A2 (OATP1A2) (Meyer zu Schwabedissen et al., 2008), the UDP-glucuronosyltransferase 1a5 (Ugt1a5), and the sulfotransferase 2a2 (Sult2a2) (Aleksunes and Klaassen, 2012). Accordingly, PXR-activation in general results in enhanced metabolic activity and increased metabolic clearance. Hyperforin even if very potent is not the only activator of PXR, multiple drugs in clinical use function as activating ligands (Meyer zu Schwabedissen and Kim, 2009). Accordingly, PXR is also called a xenosensor as it senses drug exposure and modulates

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metabolic activity in response. There are multiple examples in literature, where PXR-activation is the basis of observed drug-drug interactions, e.g. simultaneous administration of indinavir and St. John's wort reduces exposure of the protease inhibitor (Piscitelli et al., 2000). Additionally, Ruschitzka *et al.* reported heart transplant rejections in patients concomitantly treated with cyclosporine and St. John's wort (Ruschitzka et al., 2000). The authors report reduced oral bioavailability and increased hepatic clearance of the victim drug. Importantly, in order to interact with PXR and to activate transcription of the targeted genes, hyperforin has to enter the cell. So far, little is known about the transmembrane transport and cellular uptake of this molecule.

Recent findings by our group suggest that hyperforin is a substrate of OATP2B1, as observed testing whether the method of competitive counterflow (CCF) can be applied to identify substrates of this uptake transporter (Schaefer et al., 2018). OATP2B1 is a member of the organic anion transporting polypeptide family, and facilitates the sodium-independent uptake of its substrates. Since its first description (Tamai et al., 2000) multiple endogenous and exogenous substrates of the transporter have been identified (Roth et al., 2012). OATP2B1 exhibits two substrate binding sites, which can be distinguished in experimental setting by their contribution to the cellular uptake of estrone 3-sulfate (E₁S), as one is accepting E₁S at low concentration (binding site A), while the other (binding site B) mainly drives uptake at high concentrations of the sulfated steroid (Shirasaka et al., 2012). Another characteristic of OATP2B1 is its ubiquitous expression with high amounts of the transporter in brain, heart, kidney, lung, mammary gland, placenta, platelets, skeletal muscle and skin (Bronger et al., 2005; Ferreira et al., 2018; Grube et al., 2006; Knauer et al., 2010; Niessen et al., 2009; Pizzagalli et al., 2003; Sakamoto et al., 2013; Schiffer et al., 2003; St-Pierre et al., 2002). Considering the abovementioned impact of PXR on hepatic clearance and oral bioavailability it seems noteworthy, that OATP2B1 is expressed in the sinusoidal membrane of hepatocytes (Kullak-Ublick et al., 2001), and in the intestine (Keiser et al., 2017; Kobayashi et al., 2003), whereby contributing to hepatic clearance and intestinal absorption, respectively. Even if several drug transporters are part of the PXR-regulated gene network, OATP2B1 is not. (Knauer et al., 2013; Meyer zu

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Schwabedissen et al., 2018). However, our preliminary findings (Schaefer et al., 2018) suggest, that drug-drug interactions involving hyperforin may not be limited to targets of PXR, but may also involve OATP2B1 mediated uptake. It was the aim of the herein reported study to further evaluate this notion.

Materials and Methods

Cell culture. All cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO₂. The cell lines MDCKII (ATCC No. CRL-2936), HeLa (ATCC No. CCL2), HepG2 (ATCC No. HB-8065), and Caco-2 (ATCC No. HTB37) were originally obtained from the American Type Culture Collection (ATCC, Wesel, Germany) and were cultured in Dulbecco's modified Eagle Medium (DMEM, Sigma-Aldrich, Buchs, Switzerland) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich) and 1% stable glutamine (BioConcept, Basel, Switzerland). In case of Caco-2 the medium was supplemented with 1% penicillin/streptomycin (Bio-Concept, Basel, Switzerland). MDCKII-OATP2B1 cells have been established and characterized as described elsewhere (Grube et al., 2006) and were kept under continuous selection with 750 µg/ mL hygromycin B (Carl Roth, Karlsruhe, Germany).

Transport experiments. MDCKII-OATP2B1 cells were seeded in 24-well plates at a density of 50 000 cells/ well (Eppendorf, Hamburg, Germany). After one day in culture, cells were treated with sodium butyrate (2 mM) and cultured for an additional day. Uptake experiments were started washing the cells with pre-warmed phosphate buffered saline (PBS) followed by a 10-minute equilibration with Hank's balanced salt solution (HANKs, H8264, with sodium bicarbonate, without phenol red, pH 7.4, Sigma-Aldrich). To test the inhibitory potency of hyperforin (as hyperforin dicyclohexylammonium salt, Sigma-Aldrich) and hypericin (TOCRIS, Bio-Techne AG, Zug, Switzerland) cells were exposed to either estrone 3-sulfate (E₁S, 0.005 or 50 µM, Sigma-Aldrich) or 0.1 µM bromosulfophthalein (BSP, Sigma-Aldrich), supplemented with 50 000 dpm/ well of [³H]-E₁S (3 nM, Hartmann Analytic, Braunschweig, Germany), or [³H]-BSP (9 nM, Hartmann Analytic) in presence of different concentrations of the respective compound. After 5 minutes of exposure the cells were washed with ice-cold PBS, lysed in 200 µl of 0.2% SDS-5 mM EDTA and the cellular content of E₁S or BSP was quantified determining the

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amount of tracer by liquid scintillation counting using the Rotiszint eco Plus (Carl Roth) and the Tri-Carb 2900TR counter (TopLab, Basel, Switzerland). An aliquot was used to assess the amount of protein in each well using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Reinach, Switzerland) and the microplate reader Infinite® 200 Pro (Tecan, Männedorf, Switzerland). CCF experiments were performed as previously described by our group (Schaefer et al., 2018). MDCKII-OATP2B1 and MDCKII cells were seeded, treated and prepared for transport experiments as described above. Since CCF experiments are conducted in the steady state, cells were pre-incubated with [³H]-E₁S (100 000 dpm/ well) for 30 minutes. For a time dependent CCF experiment in MDCKII-OATP2B1 cells the supernatant was exchanged to either [³H]-E₁S alone or supplemented with hyperforin (5 μM) or hypericin (100 μM). Cellular accumulation of [³H]-E₁S was measured as described above after 10, 20, 30, 45, 60, 90, 120 and 180 seconds. Since the system is equilibrated again after 90 seconds this was defined as the time point when intracellular accumulation of [³H]-E₁S was measured in CCF experiments. MDCKII-OATP2B1 and MDCKII cells were treated with [³H]-E₁S until steady state before medium was changed to the same concentration of [³H]-E₁S supplemented with hyperforin, hypericin, atorvastatin (2.5 μM, Sigma-Aldrich) as positive and penicillin G (250 μM, Sigma-Aldrich) as negative control. For inhibition studies 2.5 μM atorvastatin supplemented with 50 000 dpm/ well [³H]-atorvastatin (7.5 nM, Perkin Elmer, Waltham, USA) was used. Cellular accumulation after 5 minutes of exposure was assessed in presence of different concentrations of hyperforin (0.1, 1, 5 μM) or hypericin (10, 50, 100 μM).

Cell based reporter gene assays. The previously reported CYP3A4-XREM-pGL3 plasmid was used to test the influence of OATP2B1 on PXR mediated transactivation. Briefly, HepG2 and HeLa cells were seeded in 24-well plates at a density of 50 000 cells/ well. Then the cells were transfected with 250 ng CYP3A4-XREM-pGL3 (Tirona et al., 2003), 25 ng pRL-TK, 250 ng PXR-pEF6 (Meyer zu Schwabedissen et al., 2008), 250 ng RXR-pEF6 (HeLa cells), 250 ng OATP2B1-pEF6 or 250 ng pEF6-V5/HIS as control (Thermo Fisher Scientific) using 2.25 μl/ μg DNA jetPRIME® (Polyplus distributed by Chemie Brunschwig, Basel, Switzerland). After 4 hours, the medium was changed, and the cells were kept

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in culture until treatment started 24 hours after transfection. Hyperforin, hypericin, bromosulphthalein and rifampicin were used at a final concentration of 0.1, 1, 10 and 10 μM , respectively. The formulations were tested at a 1/100 dilution of one tablet or capsule solved in 200 mL. To avoid phototoxicity the experiment was performed in the dark. Finally, the cells were lysed after 24 hours of exposure and luciferase activity was determined using the Dual-Luciferase[®] Assay System (Promega, Dübendorf, Switzerland) and the plate reader Infinite[®] M200 Pro (Tecan) as recommended by the manufacturer. The observed activity of the *firefly* luciferase was normalized to that of the *Renilla*.

Transwell transport of hyperforin and hypericin. Experiments were performed as previously described (Meyer zu Schwabedissen et al., 2018). Briefly, Caco-2 cells were seeded at a density of 3×10^5 cells/ well onto polycarbonate membranes with 0.4 μm pore size inserted in 12 well plates (Chemie Brunschwig, Basel, Switzerland) and cultivated for 14 days with medium change every second day. Measuring the transepithelial electrical resistance (TEER) value of at least 200 Ω/cm one day before the experiment confirmed the integrity of the monolayer. Furthermore, integrity was tested after the experiment by addition of 1 mg/ ml Lucifer yellow (Sigma-Aldrich) to the apical side and measuring absorbance in the basolateral compartment after 1 hour by spectrofluorometry. On the day of the experiment, cells were washed once with pre-warmed PBS before incubation with Krebs-Henseleit buffer (118 mM NaCl, 25 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 11 mM Glucose, 4.7 mM KCl) for 20 minutes at 37 °C. The buffer was adjusted to a pH of 5.5 in the apical compartment and to a pH of 7.4 in the basolateral compartment. [³H]-atorvastatin (100 000 dpm/ well) was added either to the apical or to the basolateral side to determine apical to basal (a-b) or basal to apical (b-a) permeability. 0.1 μM hyperforin was added to either the apical or the basal compartment. Amount of atorvastatin was assessed in 100 μl aliquots by scintillation counting as described above. The amount of transported [³H]-atorvastatin per time was calculated as permeability coefficient (P_{app}) as previously described (Hubatsch et al., 2007). P_{app} of apical (a) to basal (b) or b to a direction was used to calculate the uptake and efflux ratio, respectively, with the following equations:

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$$\text{uptake ratio } P_{a-b} = \frac{P_{app}(a \text{ to } b)}{P_{app}(b \text{ to } a)} \quad \text{efflux ratio } P_{b-a} = \frac{P_{app}(b \text{ to } a)}{P_{app}(a \text{ to } b)}$$

Transport studies with 11 Swiss formulations containing St. John's wort. Before transport experiments, pills were milled using the mixer mill MM 400 (Retsch GmbH, Haan, Germany) or capsules were opened to release the content. The concentration for inhibition experiments was estimated by calculating the expected concentration in the intestine when ingesting the pill or capsule with 200 ml water. Two days before the experiment MDCKII-OATP2B1 cells were seeded as described above. After washing the cells with PBS and a 10-minute pre-incubation in HANKs buffer, influence was tested by treating the cells with 0.005 μM E₁S supplemented with radiolabeled E₁S (50 000 dpm/ well) and the respective formulation diluted 1:100 and 1:1000. After 5 minutes cells were washed with ice-cold PBS and lysed in 200 μl of 0.2% SDS-5 mM EDTA. Protein amount and intracellular accumulation of [³H]-E₁S was measured as described in detail above.

Western blot analysis. HepG2 and HeLa cells were seeded at a density of 1.8×10^6 cells/ 10 cm culture dish (Eppendorf). After reaching confluence, cells were harvested in 5 mM-Tris HCl (pH 7.4) supplemented with the protease inhibitor cocktail (Sigma-Aldrich). After several cycles of freezing and thawing, protein content in the cell lysate was quantified using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were separated by SDS-PAGE (Mini-PROTEAN[®] System) followed by blotting onto a nitrocellulose membrane with the Mini Trans-blot[®] Cell (Bio-Rad Laboratories AG, Cressier, Switzerland). 5% FCS- TRIS-buffered saline -0.04% Tween 20 (TBS-T) was used to block unspecific binding before the primary antibody anti-HNF4 α (ab41898, abcam, Lucerna Chem AG, Luzern, Switzerland, diluted 1:1000) or anti-OATP2B1 ((Grube et al., 2005) diluted 1:5000) was added and incubated at 4 °C overnight. Actin (sc-1616, Santa Cruz Biotechnology, Heidelberg, Germany, diluted 1:1000) served as control. HRP-labeled secondary antibodies and the ECL Western blotting substrate (Pierce distributed by Thermo Fisher Scientific) were used to detect binding of the primary antibody. The

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Chemidoc XRS system (Bio-Rad Laboratories AG) was used for visualization and digitalization of chemiluminescence.

Determination of hyperforin and hypericin content in 11 Swiss formulations with HPLC. 100 mg of the powdered samples was dissolved in 1 ml DMSO and centrifuged at 13 000 rpm for 5 minutes before filtrating through a disposable syringe filter (Micropur PTFE, pore-size: 0.45 μ m). Dissolution of the solid residue of Deprivita[®], Hyperiplant[®], Rebalance[®], Sandoz Hypericum[®], and Vogel Hyperiforce[®] using the same procedure confirmed > 80% and > 90% dissolution of hypericin and hyperforin, respectively. Hypericin was purchased from TOCRIS (Bio-Techne AG, Zug, Switzerland) and had > 98% purity according to manufacturer. A calibration curve was prepared with a hypericin dilution ranging from 1 to 0.002 mg/ml. The hypericin calibration curve was also used for the simultaneous quantification of pseudohypericin since both compounds have the same chromophore. The identity of pseudohypericin was confirmed by chromatographic comparison with a reference sample purchased from Sigma-Aldrich. The content of hypericin was calculated as the sum of the content of hypericin and pseudohypericin. Hyperforin was purchased from Sigma-Aldrich (Buchs, Switzerland) as dicyclohexylammonium salt. According to manufacturer, purity was 98% as a mixture of hyperforin and adhyperforin. Considering that both compounds have the same chromophore and taking into account their respective molecular weights, the commercial sample was determined by HPLC-UV to contain 83.6% hyperforin and 16.4% adhyperforin. Two calibration curves were therefore prepared for hyperforin (0.9 - 0.007 mg/ml) and adhyperforin (0.18 - 0.0014 mg/ml) and the content of hyperforin was calculated as the sum of both phloroglucinols. Since hyperforin is highly sensitive to light and oxygen all preparatory steps for measuring hyperforin content were performed under exclusion of light. Prior to grinding and solving of the samples, the respective tubes were pre-filled with nitrogen to avoid oxidation. HPLC analyses were performed on an Alliance 2690 chromatographic system coupled to a PDA996 detector (Waters, Milford, MA, USA). The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% trifluoroacetic acid (TFA) (hyperforin analysis) or 0.5% TFA (hypericin analysis). Separation of

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hyperforin was achieved using a 115 Zorbax Eclipse XDB-C8 Narrow-Bore column (2.1 x 150 mm, 3.5 μ m, Agilent, Santa Clara, CA, USA) with a gradient of 50% to 100% B in 20 minutes, then 100% B for 15 minutes at a flow rate of 0.4 ml/ minute. Hypericin was analyzed on an Ascentis Express C18 column (3 x 100 mm, 2.7 μ m, Supelco, Bellefonte, PA, USA) with a gradient of 45 to 100% in 15 minutes, then 100% B for 5 minutes at a flow rate of 0.6 ml/ minute. The injection volume was 10 μ l for the calibrators and the formulation samples. Detection was at 272 nm (hyperforin) or 588 nm (hypericin). Separations were performed at 30 °C. Data are reported as mean \pm SD of three independent analyses.

Statistical analysis. Microsoft Excel (Microsoft, Redmond, USA) and GraphPad Prism software 6.0 (GraphPad Software, San Diego, USA) was used to analyze the herein reported data sets. Tests used for statistical analysis are described in the context of data presentation. A *p*-value below 0.05 was considered statistically significant.

Results

Interaction of hyperforin and hypericin with OATP2B1 mediated uptake. In order to validate the interaction of hyperforin and hypericin with OATP2B1 their impact on the cellular accumulation of the known substrates estrone 3-sulfate (E₁S) (Pizzagalli et al., 2003) and bromosulfophthalein (BSP) (Kullak-Ublick et al., 2001) was determined in MDCKII-OATP2B1 cells. As shown in Figure 1 the estrone 3-sulfate inhibition studies accounted for the previously reported two binding sites (Shirasaka et al., 2012) testing the influence of hyperforin and hypericin on estrone 3-sulfate (E₁S) accumulation at low (Figure 1A and 1D; representing binding site A) and at high concentrations (Figure 1B and 1E, representing binding site B). For hyperforin we observed concentration dependent inhibition for both binding sites recognizing E₁S. The inhibition data were basis for an estimation of the respective IC₅₀ values revealing highest potency for binding site A mediated transport of E₁S with an IC₅₀ value of 0.32 μ M (CI 0.24 to 0.42 μ M, Figure 1A), while for binding site B the IC₅₀ value was 5.55 μ M (CI 2.10 to 14.73 μ M). Inhibition of OATP2B1 mediated cellular accumulation of BSP resulted in an estimated IC₅₀ value for hyperforin of 0.82 μ M (CI 0.53 to 1.26 μ M). Similar results were obtained for hypericin, even if this

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compound exhibited much lower IC_{50} values for binding site A (20.79 μ M; CI 13.90 to 31.10 μ M, Figure 1D), binding site B 256.2 μ M (CI 119.4 to 550.0 μ M, Figure 1E), and for BSP (108.7 μ M (CI 76.39 to 154.8 μ M, Figure 1E). In order to test whether hyperforin and hypericin are also substrates of OATP2B1 we applied the method of competitive counterflow (CCF). The basis of this experimental procedure used to identify substrates competing for substrate recognition is depicted in Figure 1G. Briefly, interaction with the transporter is assessed in the steady state. Reduction of the intracellular estrone 3-sulfate amount in the equilibrium is due to competitive inhibition showing that the test compound is a substrate of the facilitating mechanism, while inhibitors are not influencing the cellular equilibrium (Harper and Wright, 2013). As shown in Figure 1H presence of the known OATP2B1 substrate atorvastatin, as well as hyperforin (5 μ M) or hypericin (100 μ M) reduced the amount of E_1S in the steady state ($p < 0.05$), whereby suggesting that both constituents of St. John's wort are not only inhibitors, but also substrates of OATP2B1. Penicillin G served as non-substrate control (Schaefer et al., 2018). When performing CCF experiments in MDCKII cells no influence on the cellular amount of E_1S was observed for any of the herein tested compounds (Supplemental Figure 1). Assessing the time dependency of reaching the second equilibrium for hyperforin (Supplemental Figure 2A) and hypericin (Supplemental Figure 2B) revealed that it is reached after 90 seconds of exposure, which is comparable to that previously reported (Schaefer et al., 2018).

Influence of OATP2B1 on hyperforin mediated transactivation of CYP3A4. Hyperforin is a well-known ligand and activator of the human Pregnane X Receptor (PXR) whereby influencing transcription of CYP3A4 (Moore et al., 2000). In order to verify that hyperforin is a substrate of OATP2B1 we applied cell based reporter gene assays assessing the transactivation of a synthetic CYP3A4-XREM-pGL3 reporter gene construct. As shown in Figure 2A HepG2-cells transfected with PXR- and OATP2B1-pEF6 exhibited higher luciferase activation ($p < 0.05$) than cells not heterologously expressing the transporter. Testing the influence of 10 μ M BSP on hyperforin (0.1 μ M) induced PXR-mediated transactivation of CYP3A4 in OATP2B1-overexpressing cells showed that presence of this competitive inhibitor of the

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transporter reduced luciferase activity statistically significantly. Importantly, BSP did not influence the luciferase activity (Figure 2B).

Impact of hyperforin and hypericin on transcellular fluxes in Caco-2 cells. Expression of OATP2B1 is assumed to contribute to intestinal absorption of orally applied drugs. Transcellular flux experiments using the intestinal model cell line Caco-2 cells are often applied in the preclinical phase to not only predict bioavailability, but also to determine whether there is an active transport component influencing the transcellular path of a new molecular entity. OATP2B1 is expressed in Caco-2 cells and is part of the network of transporter influencing the transcellular flux (Meyer zu Schwabedissen et al., 2018). In order to determine whether hyperforin influences transcellular fluxes, and thereby oral bioavailability, Transwell® experiments were performed using the known OATP2B1 substrate drug atorvastatin (Grube et al., 2006). At first, interaction of hyperforin with OATP2B1 mediated atorvastatin uptake was tested (Figure 3A) revealing a decreased amount of intracellular atorvastatin with increasing concentrations ($p < 0.05$). As controls, BSP and E₁S were included in the transport inhibition study. Estimation of the inhibitory potency, even if based on a limited number of data points, suggested an IC₅₀ value for hyperforin of 0.23 μM (CI 0.11 to 0.48 μM). As shown in Figure 3B, hyperforin increased the efflux ratio observed for atorvastatin (mean efflux-ratio ± SD; hyperforin vs. DMSO; 2.863 ± 0.415 vs. 1.828 ± 0.332 ; $p = 0.028$). The observed effect can mainly be attributed to changes in the flux from apical (a) to basal (b) as there was a trend towards lower movement in this direction (mean P_{app} (a-b) ± SD (cm/ s); hyperforin vs. DMSO; $1.33 \times 10^{-6} \pm 3.161 \times 10^{-7}$ vs. $1.83 \times 10^{-6} \pm 2.621 \times 10^{-7}$; $p = 0.103$, Figure 3C). No trend for a change was observed for the atorvastatin flux in b to a direction (mean P_{app} (b-a) ± SD (cm/ s); hyperforin vs. DMSO $3.81 \times 10^{-6} \pm 1.153 \times 10^{-6}$ vs. $3.36 \times 10^{-6} \pm 8.502 \times 10^{-7}$; $p = 0.615$, Figure 3D). Taken together these data suggest that hyperforin interacts with intestinal absorption of atorvastatin, which is in part mediated by OATP2B1.

Effect of St. John's wort formulations on OATP2B1-mediated transport. St. John's wort is widely used as medication to treat mild to moderate depression. In Switzerland there are currently 11 solid

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formulations marketed. These formulations contain different amounts of hypericin and hyperforin as reported by the manufacturer and summarized in Table 1. In a screening experiment we tested for interaction of the formulations with OATP2B1 mediated E₁S uptake in MDCKII-OATP2B1 cells. This screening was conducted using two concentrations of the respective formulation, representing a 1/100 or 1/1000 dilution of one tablet or capsule solved in 200 mL liquid. As shown in Figure 4A, none of the 1/1000 dilutions lowered the cellular accumulation of E₁S compared to solvent control ($p > 0.05$). However, for the formulations Arkocaps[®], Deprivita[®], Hänseler Menopause[®], Hyperiplant[®], Jarsin[®], Sandoz Hypericum[®] and Solevita[®] we observed reduced OATP2B1-mediated uptake of estrone 3-sulfate for the 1/100 dilution ($p < 0.05$). Rebalance[®], Remotiv[®], Vogel Hyperimed[®] and Vogel Hyperiforce[®] did not affect OATP2B1 mediated E₁S uptake in our experiments.

Subsequently, we assessed the influence of the respective formulation on the PXR-mediated transactivation of CYP3A4 either in HepG2 (Figure 4B), or HeLa (Figure 4C). Notably, in accordance to our transport inhibition experiments Rebalance[®], Remotiv[®], Vogel Hyperimed[®] and Vogel Hyperiforce[®] were not affecting luciferase activation in either cell-based reporter gene assay ($p > 0.05$). Interestingly, treatment of HepG2 cells resulted in an up to 125-fold induction in presence of a 1/100 dilution of the formulations, while a maximum of about 7-fold induction was observed in HeLa cells. This might be explained by the endogenous expression of HNF4 α , which is permissive for the transactivation of CYP3A4 by PXR (Tirona et al., 2003). The lack of HNF α expression in HeLa cells was verified by Western blot analysis (Figure 4D; mean HNF4 α expression normalized to actin \pm SD; HepG2 vs. HeLa; 17.88 ± 3.43 vs. 0.26 ± 0.02 ; $p < 0.05$, $n=3$, unpaired t-test with Welch's correction), while no difference in expression of OATP2B1 was observed (Figure 4E; mean OATP2B1 expression normalized to actin \pm SD; HepG2 vs. HeLa; 1.52 ± 1.28 vs. 1.41 ± 0.72 ; $p > 0.05$, $n=3$, unpaired t-test with Welch's correction).

Quantification of hyperforin and hypericin content in the St. John's wort formulations. As mentioned before, most manufacturers merely provide information on the content of hypericin in their formulation due to the primal assumption that this component determines biological activity of the extract.

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We quantified the amount of hyperforin (as the sum of hyperforin and adhyperforin) and hypericin (as the sum of hypericin and pseudohypericin) in each formulation by HPLC. As summarized in Table 1 and depicted in Supplemental Figure 3 and 4, all formulations contained hypericin, while hyperforin was detected in all medications but Vogel Hyperimed[®] and Vogel Hyperiforce[®] and to a low extent in Rebalance[®] and Remotiv[®]. Importantly, these four medications neither interacted with OATP2B1 mediated E₁S accumulation nor did they transactivate CYP3A4 in the herein reported cell-based reporter gene assays, suggesting that hyperforin is the key determinant of the observed effects. In order to test this notion we analyzed whether there is a correlation between hyperforin-content and the observed luciferase activation assessed in transfected HepG2 (Figure 5A), or HeLa cells (Figure 5B) revealing a direct correlation. The association as determined by Pearson-correlation was most pronounced when excluding Hyperiplant[®] the formulation containing the highest amount of hyperforin. An indirect and statistically significant correlation was observed for the percentage of E₁S uptake and hyperforin content in MDCKII-OATP2B1 cells (Figure 5C). Importantly, no correlation was observed for hypericin and the experimental results (Figure 5D-F).

Discussion

In this study we report that the constituents of St. John's wort - hyperforin and to a lower extent hypericin - are inhibitors of the ubiquitously expressed membrane transporter OATP2B1. Furthermore, CCF experiments suggested that the compounds are not only inhibitors but also substrates of OATP2B1. This was further supported by findings in reporter gene assays where OATP2B1 enhanced hyperforin-induced transactivation of CYP3A4, which was reduced in presence of the competitive inhibitor bromosulfophthalein (BSP).

Especially for hyperforin, it is known, that it is a major determinant of drug-drug interactions observed during co-administration of St. John's wort, where a pronounced increase in CYP3A4 activity has to be expected (Willson and Kliewer, 2002). However, the influence of concomitant use of St. John's wort is not limited to CYP3A4-substrates as the underlying mechanism, which is the binding and activation of

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PXR finally resulting in induced expression of multiple target genes, most of them with function in drug elimination (Tolson and Wang, 2010). Indeed, one compound exemplifying this is the cardiac glycoside digoxin (Johne et al., 1999). This drug is commonly used in clinics, and is not a CYP3A4 substrate (Lacarelle et al., 1991), but findings by Greiner *et al.* clearly showed that concomitant oral intake of digoxin and rifampin decreased drug disposition and exposure due to induction of P-glycoprotein (P-gp, ABCB1, MDR1) expression (Greiner et al., 1999). Importantly, rifampin is another well-known activating-ligand of PXR, and can be applied to analyze the influence of PXR-activation on drug metabolism (Chen and Raymond, 2006). Accordingly, the reduction in oral bioavailability of digoxin can be explained by enhanced expression of ABCB1 (Greiner et al., 1999). This transporter is responsible for active extrusion of substrates from enterocytes back into the intestinal lumen (Shapiro and Ling, 1995). Considering our findings on inhibition of OATP2B1-mediated transport by hyperforin and hypericin, the interaction of St. John's wort is extended to a further class of compounds – namely OATP2B1 substrates, where the mechanism of interaction would be most likely the competitive inhibition of cellular uptake.

OATP2B1 is assumed to play a role in intestinal absorption of orally administered drugs (Shitara et al., 2013). Testing the influence of hyperforin on transcellular transport of atorvastatin revealed an enhanced efflux ratio for atorvastatin. The experimental set-up of Caco-2 cells cultured in Transwells[®] is commonly applied for predictions on bioavailability and the contribution of transporters to intestinal absorption (Hubatsch et al., 2007). Indeed, an efflux ratio, which is calculated relating the net flux in b to a direction to that in a to b direction, above 1.5 is assumed to indicate that active transporter-mediated processes are involved in the transcellular transport of a compound (Hubatsch et al., 2007). In our study, one is certainly tempted to attribute the observed effect of an enhanced efflux ratio to the inhibition of OATP2B1 only. However, assessed are net fluxes, and atorvastatin is even if it is a reported OATP2B1 substrate (Grube et al., 2006) not a specific substrate of this transporter. Indeed, atorvastatin is also a substrate of the efflux transporters multidrug resistance-associated protein (MRP)1 (ABCC1), MRP4 (ABCC4), MRP5 (ABCC5) (Knauer et al., 2010), and ABCB1 (Wu et al., 2000). Especially for ABCB1 there are data

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suggesting that there might also be an influence of hyperforin on direct transport activity (Hennessy et al., 2002). Our data suggest that presence of hyperforin acutely interacts with oral absorption of OATP2B1 substrates, enhancing the complexity of drug-hyperforin interactions that should be considered, when evaluating the interaction potential of St. John's wort preparations.

In addition to its involvement in intestinal drug absorption, OATP2B1 is assumed to contribute to the hepatocellular uptake. Recent findings by Lutz *et al.* suggest that there is at least some induction of OATP-mediated transport, as observed for concomitant treatment with rosuvastatin and pravastatin and increasing doses of rifampin (Lutz et al., 2018). Even if OATP2B1 (OATP-B) may contribute to the hepatocellular uptake of these statins (Knauer et al., 2010; Shirasaka et al., 2010), OATP2B1 is not regulated in primary human hepatocytes treated with the PXR-inducer (Jigorel et al., 2006). Although there is no direct regulation by PXR, we have recently reported interaction of the transporter with PDZK1, which influences OATP2B1 membrane localization (Ferreira et al., 2018). PDZK1 is transcriptionally controlled by multiple nuclear receptors (Ferreira et al., 2017; Prestin et al., 2017). Accordingly, whether long-term exposure to hyperforin influences localization of OATP2B1 remains to be determined, as we only tested the acute influence. Our findings suggest that the intracellular abundance of hyperforin, and thereby PXR-mediated transactivation of CYP3A4, is influenced by OATP2B1. Considering this finding, one might speculate that changes in OATP2B1 activity could influence the extent of PXR activation. Multiple mechanisms affect the activity of a transporter, one possibility is the change in expression by transcriptional modifiers, another would be modifications in genetic information with influence on transport activity. We have recently reported that OATP2B1 is transcriptionally modulated by thyroid hormones (Meyer zu Schwabedissen et al., 2018), whereby associating thyroid hormone status to OATP2B1 transport function, while Nozawa *et al.* identified genetic polymorphisms, which changed the maximal transport velocity (V_{\max}) of OATP2B1 (Nozawa et al., 2002).

Testing extracts from all oral formulations of St. John's wort currently marketed in Switzerland for their influence on OATP2B1 transport activity, and the influence of OATP2B1 on their transactivating activity

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revealed a clear association between hyperforin content and the observed effect for most formulations. Importantly, no such association was observed for hypericin content and PXR-mediated transactivation, whereby validating that hypericin is no activator of PXR (Moore et al., 2000; Wentworth et al., 2000). Furthermore, there is no clear association of hypericin content and OATP2B1 inhibition, which is in line with the much lower inhibitory potency observed for hypericin compared to hyperforin. This indicates that hyperforin is the component of St. John's wort extracts being involved in interaction with OATP2B1 mediated uptake.

The formulations were tested in cell-based reporter gene assays using HepG2 or HeLa cells as cellular model. In the liver cell model we observed a huge inducing of luciferase activity of about 50-100-fold while the increase in activity was only about 5-7-fold in HeLa cells with some formulations not affecting transactivation. On the one hand, the difference in extent of activation might certainly be explained by endogenous expression of HNF4 α in HepG2 cells, which is not present in HeLa cells as confirmed by Western blot analysis in this study and previously reported by Knauer *et al.* (Knauer et al., 2013). Importantly, HNF4 α is permissive for the transcriptional regulation by PXR (Ma et al., 2008). On the other hand, the fact that we observed induction in HeLa cells and a clear association of the extent of activation with the hyperforin content suggests that hyperforin is the driving component, but also that there is at least some HNF4 α independent PXR activation.

As mentioned before, the hyperforin content was directly correlated with the observed luciferase activation. However, this association was most pronounced when excluding Hyperiplant[®] from the correlation. With other words, the observed effect of Hyperiplant[®] on OATP2B1-mediated transport or CYP3A4 activation was lower than expected considering the hyperforin content. In this context it seems noteworthy that hyperforin is phototoxic (Onoue et al., 2011), inducing acute cell lysis. Even if our experiments were conducted under light protection, we cannot exclude that some toxicity was present modifying the experimental outcome.

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Taken together we report that hyperforin is a potent inhibitor of OATP2B1 mediated cellular uptake and we suggest that it is also a substrate. Inhibition of the transporter may influence the contribution of OATP2B1 to pharmacokinetics of its substrate drugs. Moreover, OATP2B1 activity influences intracellular accumulation of hyperforin, and thereby its binding to the xenosensor. With this study, we added to the complexity of potential mechanisms that should be included in the evaluation of potential drug-drug interactions associated with the dispensing of St. John's wort.

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The herein reported study will be part of the PhD thesis of Anima M. Schaefer.

Author Contributions:

Participated in research design: Schaefer, Potterat, Meyer zu Schwabedissen

Conducted experiments: Schaefer, Potterat, Seibert, Fertig, Meyer zu Schwabedissen

Contributed analytical tools: Meyer zu Schwabedissen, Potterat

Performed data analysis: Schaefer, Potterat, Meyer zu Schwabedissen

Wrote or contributed to the writing of the manuscript: Schaefer, Potterat, Meyer zu Schwabedissen

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. None of the authors has a conflict of interest to declare.

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Footnotes

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Figure Legends

Figure 1. Interaction of hyperforin and hypericin with OATP2B1. Inhibition of OATP2B1 mediated transport by hyperforin and hypericin was tested in MDCKII-OATP2B1 cells. Determined was the influence on the cellular accumulation of estrone 3-sulfate (E₁S) at low (0.005 μM; **A** and **D**) or high (50 μM; **B** and **E**) concentration to account for binding site A and B, respectively, or of bromosulphophthalein (BSP; **C** and **F**). The IC₅₀ values were calculated fitting the data to a sigmoidal log(inhibitor)-normalized response curve, without constraining of top or bottom, or weighting. **G** presents a schematic depicting the molecular basis of competitive counterflow (CCF) experiments, where substrates are reducing the amount of another substrate in the steady state. Tested was the influence of hyperforin or hypericin on the cellular equilibrium of E₁S in MDCKII-OATP2B1 cells compared to DMSO control (**H**). Atorvastatin and penicillin G were used as positive and negative controls, respectively. Data are presented as mean ± SD of n=3 independent experiments each performed in biological triplicates. **p* ≤ 0.05 One-way ANOVA with Dunnett's multiple comparisons test.

Figure 2. Influence of OATP2B1 on hyperforin induced PXR mediated transactivation of CYP3A4. Luciferase activation was determined in HepG2 cells transfected with PXR-pEF6 in presence or absence of heterologously expressed OATP2B1 and treated with 0.1 μM hyperforin or DMSO (striped bars) (**A**). The influence of bromosulphophthalein on hyperforin induced PXR activation was assessed in HepG2 cells transiently transfected with PXR-pEF6 and OATP2B1-pEF6 (**B**). *Firefly* luciferase activity was normalized to that of *Renilla*. Data are presented as mean ± SD of n=3 independent experiments each performed in biological triplicates. **p*-value ≤ 0.05, Two-way ANOVA with Tukey's multiple comparisons test.

Figure 3. Interaction of hyperforin with OATP2B1 mediated transport of atorvastatin. The inhibitory effect of hyperforin on atorvastatin transport was determined in MDCKII-OATP2B1 cells, bromosulphophthalein (BSP) and estrone 3-sulfate (E₁S) served as control (**A**). The influence on transcellular fluxes was assessed in Transwell® experiments using cultured Caco-2 cells. The

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unidirectional P_{app} -coefficients in apical (a) to basal (b) (C), or b to a direction (D) for atorvastatin were used to calculate the efflux ratio (P_{app} (b-a)/ P_{app} (a-b), B). Data are presented as mean \pm SD of n=3 independent experiments each performed in biological triplicates, * $p \leq 0.05$ One-way ANOVA with Dunnett's multiple comparisons test (A) or Student's *t*-test (B, C, D).

Figure 4. Influence of commercially available St. John's wort preparations on OATP2B1 function and transactivation of CYP3A4. Transport inhibition studies assessing binding site A were conducted in MDCKII-OATP2B1. Formulations were tested in two concentrations representing a 1/100 or 1/1000 dilution of the respective formulation solved in 200 mL liquid (A). The influence of each formulation (1/100 dilution) in CYP3A4-activation was determined in cell based reporter gene assays using HepG2 (B) or HeLa cells as cellular model (C). *Firefly* luciferase was normalized to that of *Renilla* in each sample. Expression of HNF4 α and OATP2B1 in HepG2 and HeLa cells was detected by Western blot analysis, actin served as control (D, E). Data are presented as mean \pm SD of n=3 independent experiments. * p -value ≤ 0.05 One-way ANOVA followed by Dunnett's multiple comparisons test; Vogel 1 = Hyperimed[®]; Vogel 2 = Hyperiforce[®]

Figure 5. Association of hyperforin or hypericin content with the observed effects in cell based reporter gene assays, or transport inhibition studies. Luciferase activity was determined in transiently transfected HepG2 (A, D) or HeLa cells (B, E). Inhibition studies were conducted in MDCKII stably expressing OATP2B1 (C, F). Association of the observed effect with hyperforin or hypericin content was analyzed by linear regression (indicated by dotted line, shown with confidence interval), correlation was determined calculating the Pearson-coefficient. Data are presented as mean \pm SD with results of n=3 independent experiments performed in biological triplicates. R = Pearson-coefficient; open symbol indicated "Hyperiplant[®]", which was neither included in the linear regression nor the calculation of the Pearson-coefficient.

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Tables

Table 1. Content of hypericin and hyperforin determined by HPLC-UV. Percentage of hypericin or hyperforin content given in the compendium was converted to content per 100 mg extract. For quantification by HPLC a standard curve was used to calculate the concentration of hypericin or hyperforin, respectively. Data from quantification are of three independent experiments.

Formulation (Tradename)	Hypericin*	Hypericin*		Hyperforin**	Hyperforin**	
	(declared content)	(quantified by HPLC)		(declared content)	(quantified by HPLC)	
	mg/ 100 mg	mg/ 100 mg	± SD	mg/ 100 mg	mg/ 100 mg	± SD
ARKOCAPS®	0.3	0.09	± 0.04	-	0.24	± 0.01
DEPRIVITA®	0.1-0.3	0.18	± 0.01	-	1.04	± 0.05
HAENSELER MENOPAUSE®	0.1-0.3	0.10	± 0.01	-	0.34	± 0.01
HYPERIPLANT®	0.1-0.3	0.15	± 0.02	3-6	1.62	± 0.03
JARSIN®	0.1-0.3	0.20	± 0.02	-	0.70	± 0.02
REBALANCE®	0.1-0.3	0.08	± 0.01	< 0.2	0.02	± 0.01
REMOTIV®	0.1-0.3	0.09	± 0.01	< 0.2	0.02	± 0.01
SANDOZ HYPERICUM®	0.1-0.3	0.21	± 0.02	-	0.85	± 0.04
SOLEVITA®	0.1-0.3	0.16	± 0.03	-	1.02	± 0.07
VOGEL HYPERIMED®	0.5-0.8	0.08	± 0.01	-	-	-
VOGEL HYPERIFORCE®	0.2-2.1	0.08	± 0.01	-	-	-

*The content of hypericin corresponds to the sum of the content of hypericin and pseudohypericin; **The content of hyperforin corresponds to the sum of the content of hyperforin and adhyperforin.

Figure 1

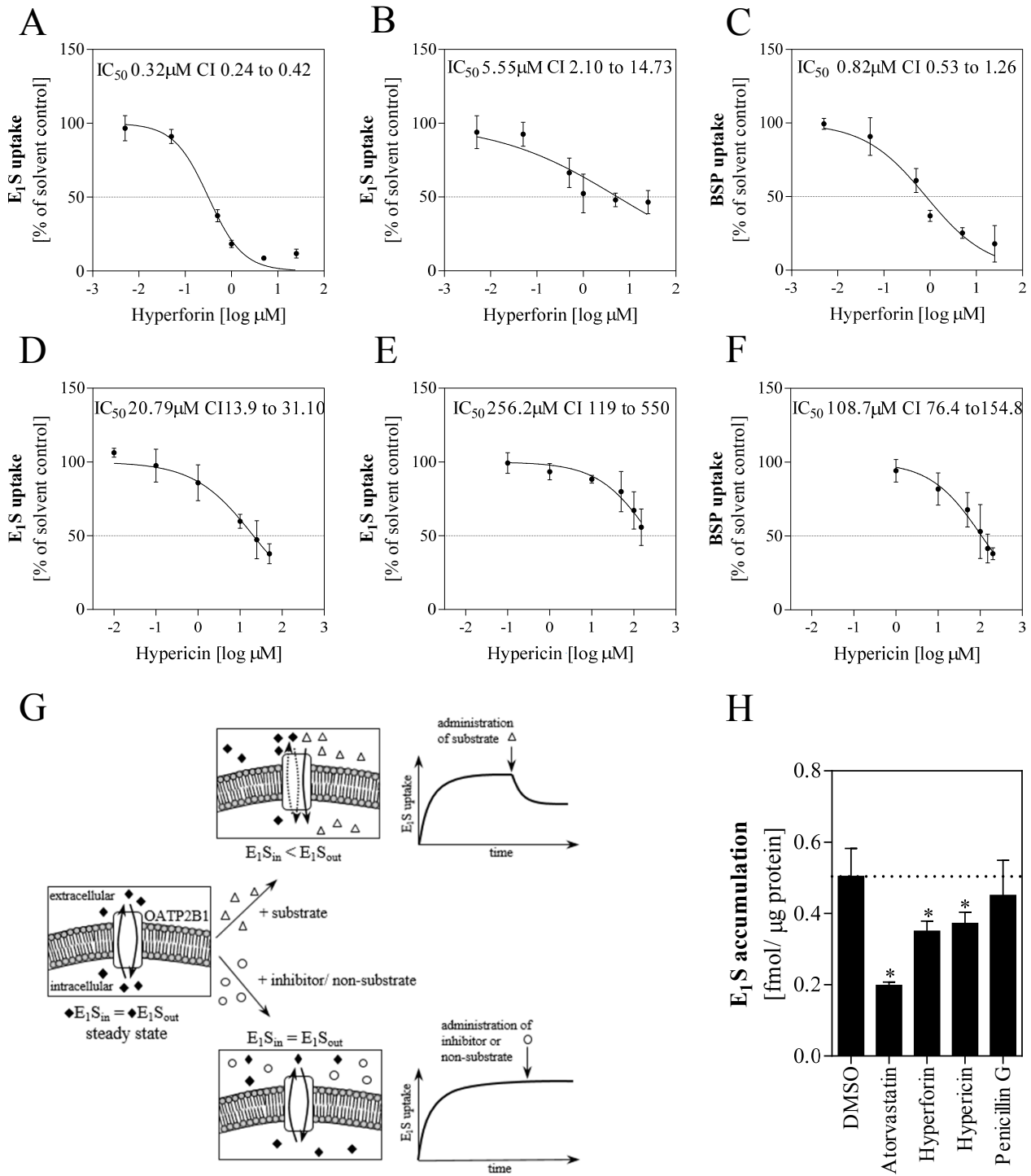


Figure 2

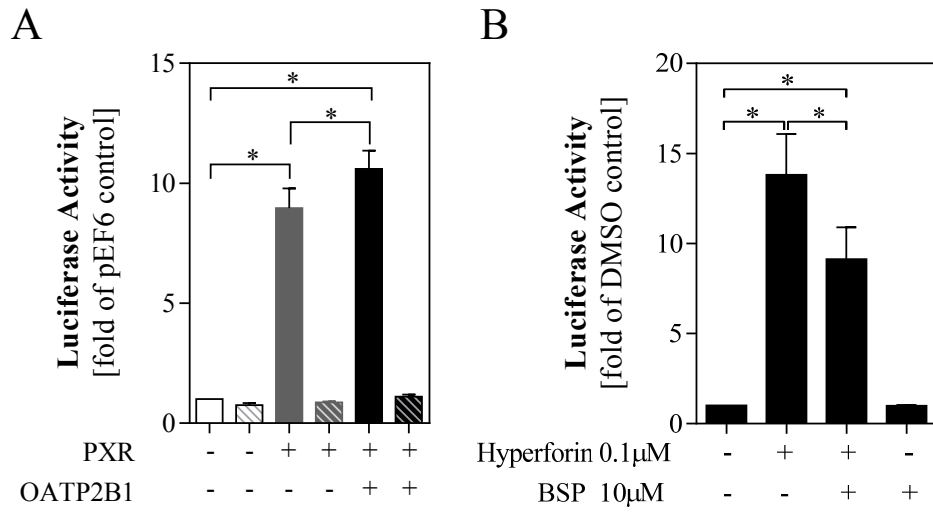


Figure 3

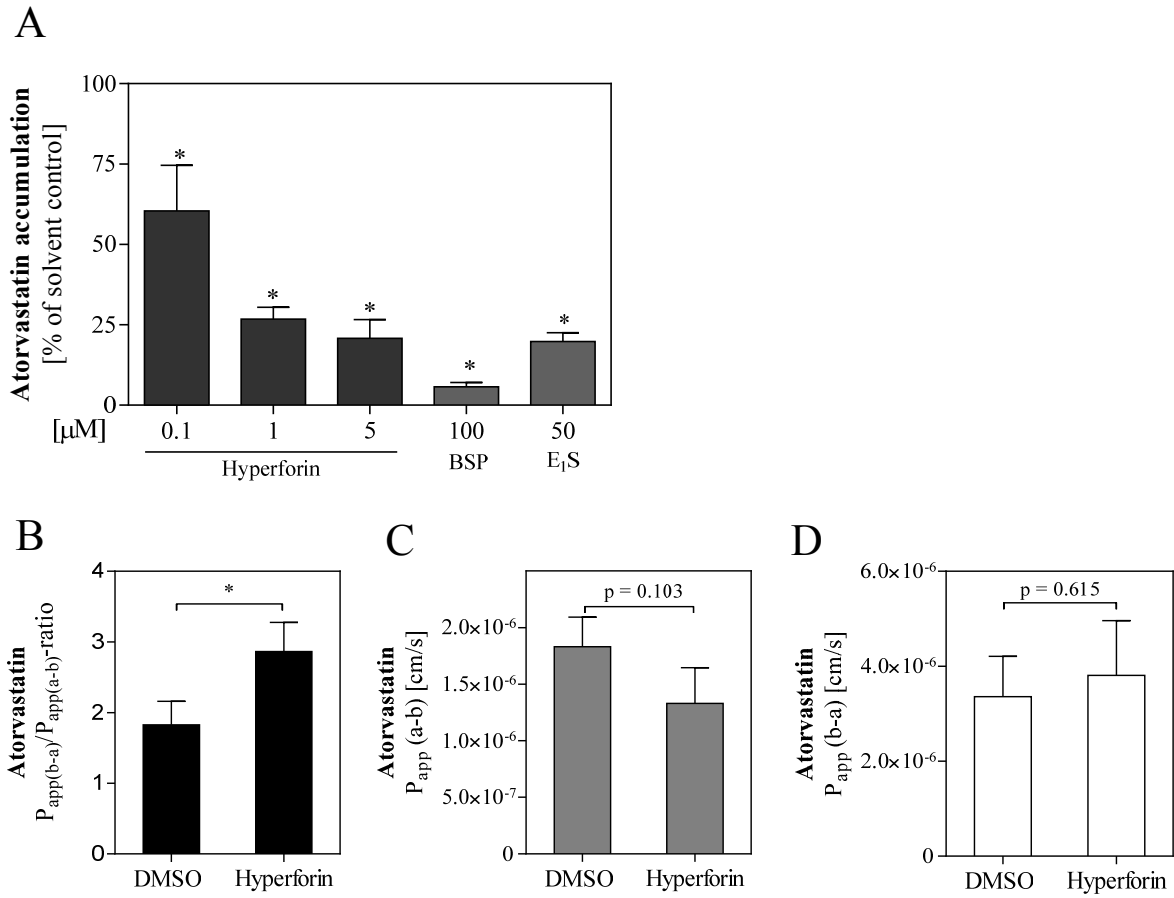


Figure 4

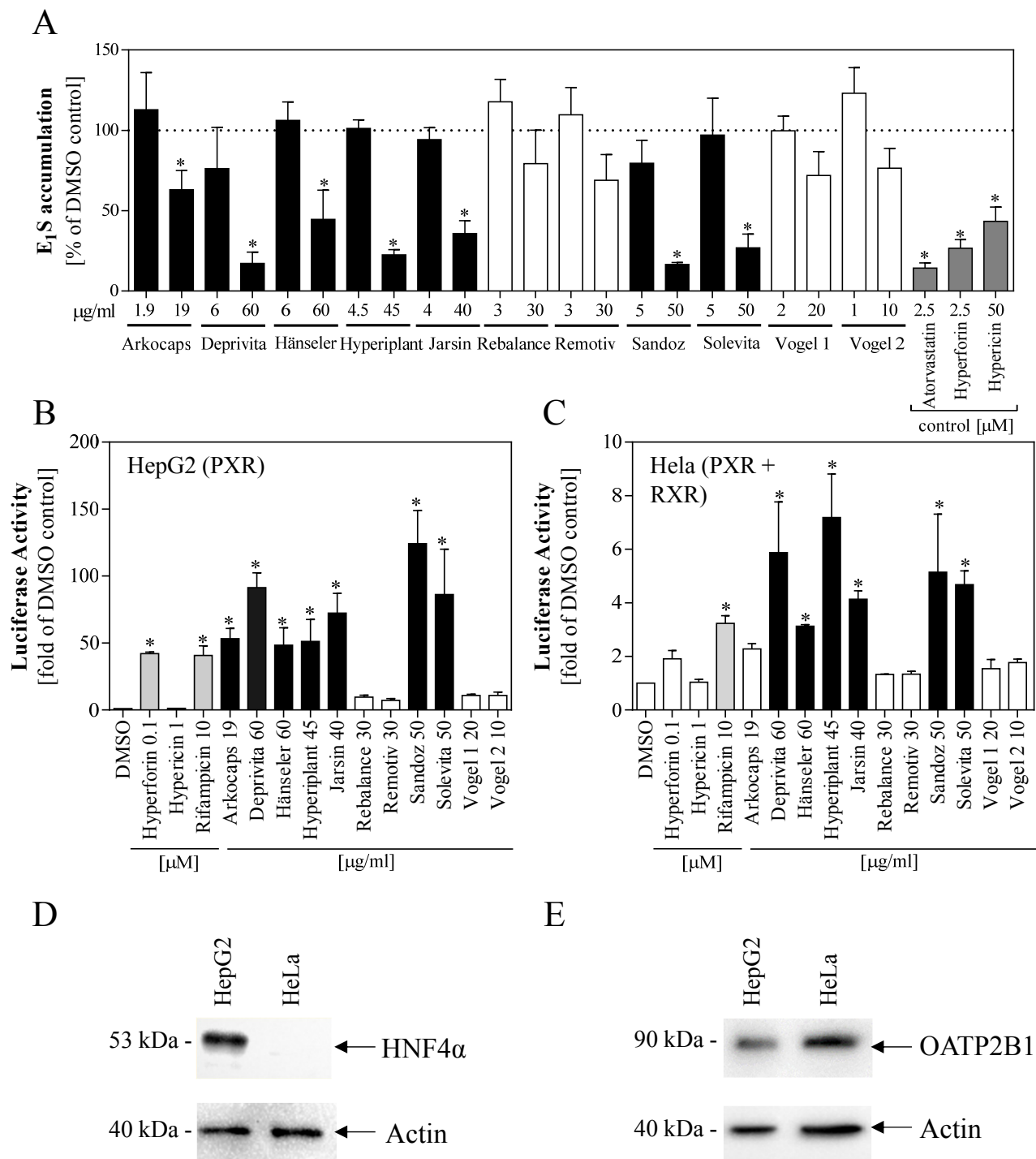
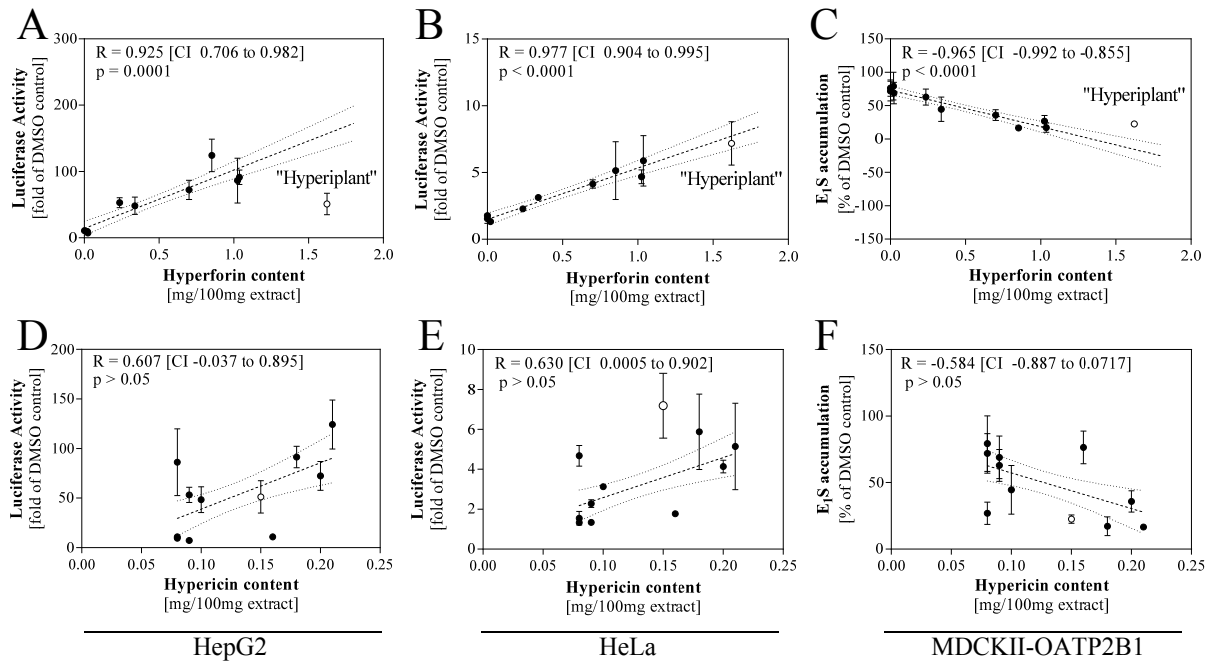


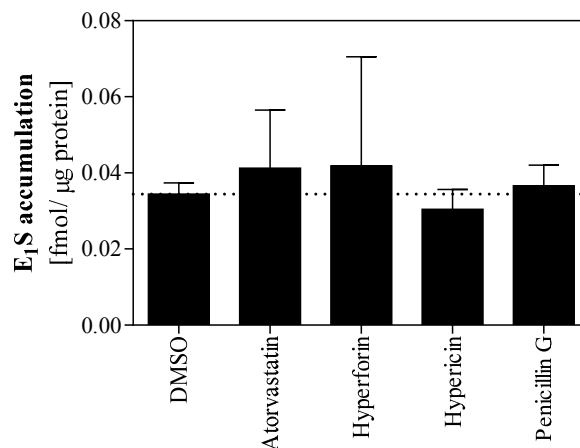
Figure 5



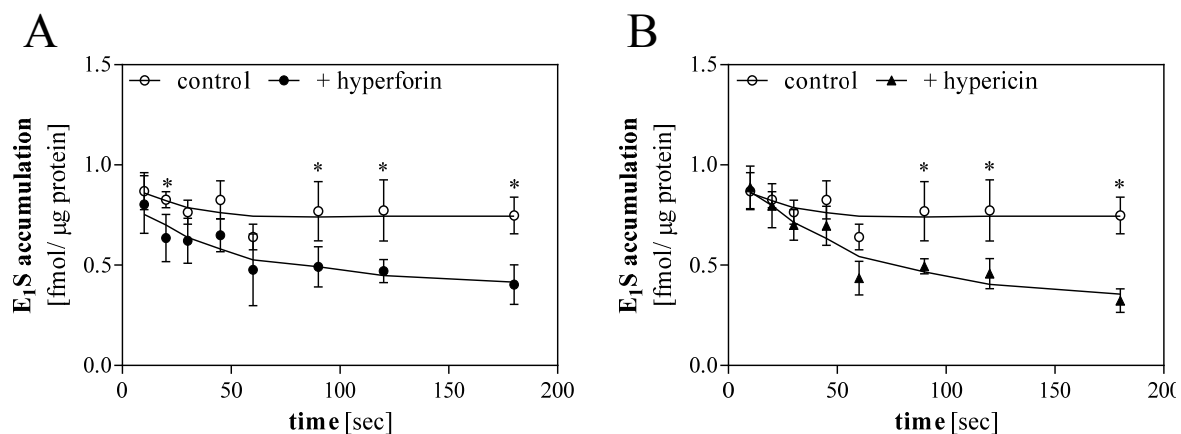
Hyperforin induced activation of the Pregnane X Receptor is influenced by the Organic Anion Transporting Polypeptide 2B1 (OATP2B1)

Anima M. Schäfer, Olivier Potterat, Isabell Seibert, Orlando Fertig, Henriette E. Meyer zu Schwabedissen

- Supplemental Data -

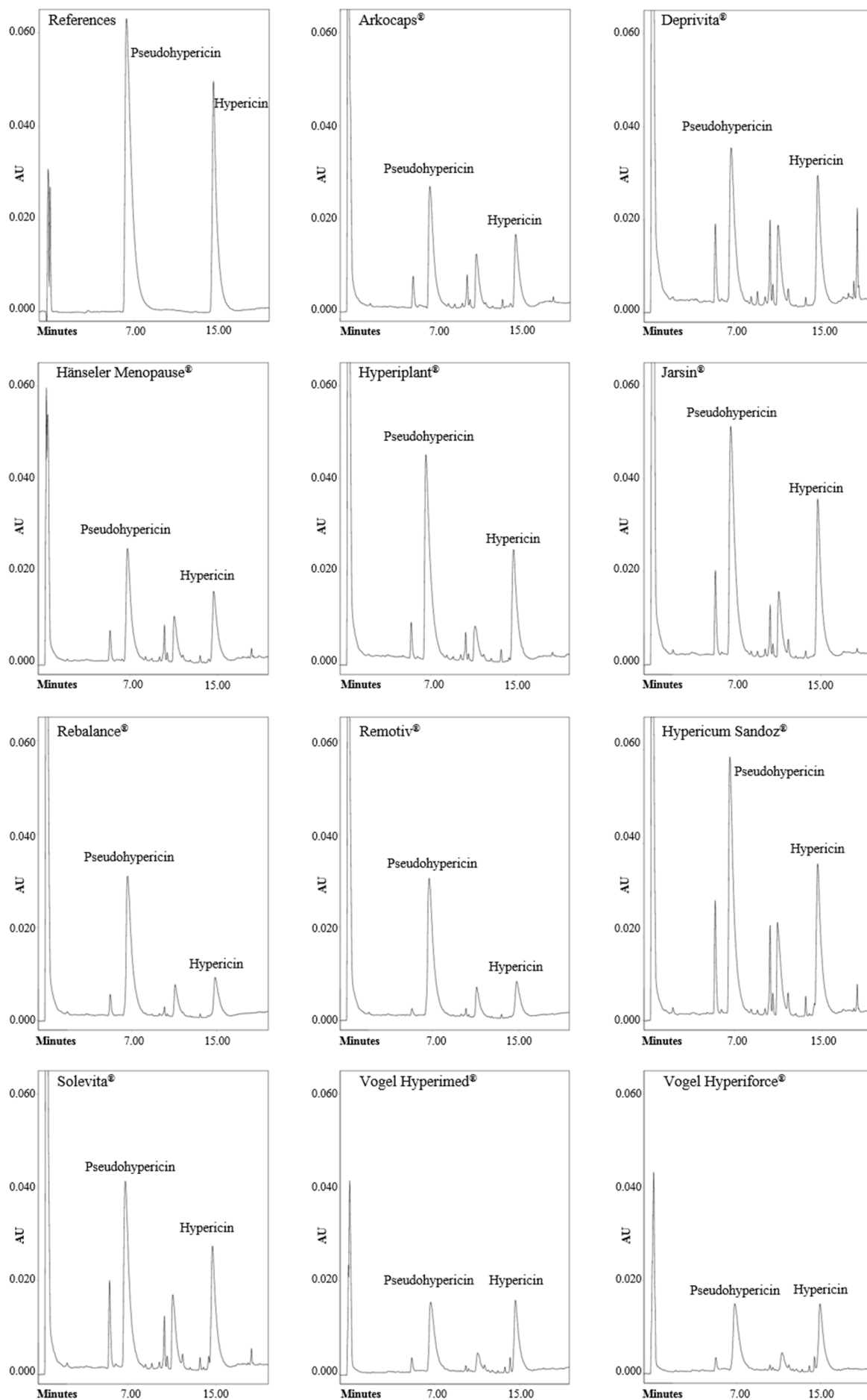


Supplemental Figure 1. Counterflow experiments performed in MDCKII cells. Cells were exposed to [³H]-E₁S for 30 min. After reaching the steady state the supernatant was replaced by [³H]-E₁S supplemented with DMSO or the respective test compound. Atorvastatin was used as positive control, penicillin G served as negative control, DMSO was the solvent control. Data are presented as mean ± SD of n=3 independent experiments each performed in biological triplicates. For statistical analysis one-way ANOVA was used corrected for multiple comparisons (Dunnett's test).



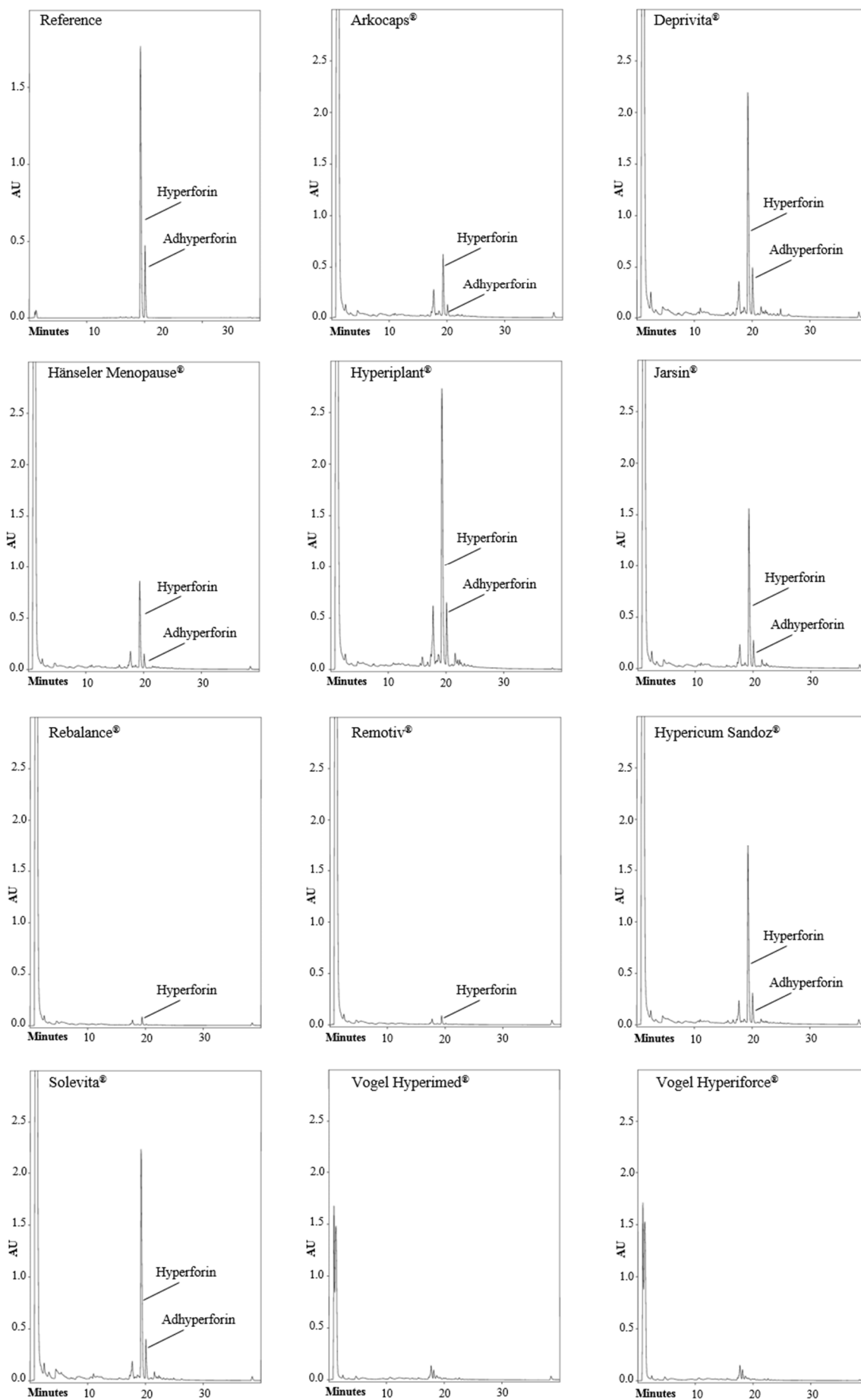
Supplemental Figure 2. Competitive counterflow experiment with hyperforin (A) or hypericin (B). MDCKII-OATP2B1 cells were treated with [3 H]- E_1S for 30 min to reach steady state. Then the supernatant was removed and cells were exposed to either the same amount of [3 H]- E_1S (control) or the same amount of [3 H]- E_1S supplemented with either hyperforin (0.5 μ M, A) or hypericin (100 μ M, B). Cellular accumulation of the radiolabel was quantified at the respective time points by liquid scintillation counting. Data are presented as mean \pm SD, of $n=3$ independent experiments performed in biological duplicates followed by nonlinear curve fitting with Savitsky-Golay smoothing. * $p \leq 0.05$, two-way ANOVA with Sidak's multiple comparisons test.

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Supplemental Figure 3. Chromatograms of a mixture of the references hypericin (TOCRIS) and pseudohypericin (Sigma-Aldrich), and of the 11 in Switzerland marketed St. John's wort formulations. Detection was at 588 nm. Experiments were performed n=3 with three independent experiments, one representative chromatogram is shown.

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Supplemental Figure 4. Chromatograms of the reference hyperforin (Sigma-Aldrich) and of the 11 in Switzerland marketed St. John's wort formulations. Detection was at 272 nm. Experiments were performed n=3 with three independent experiments, one representative chromatogram is shown.