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Circadian Variations in Exsorptive Transport: In Situ Intestinal Perfusion Data and In Vivo Relevance

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The circadian timing system (CTS) governs the 24-h rhythm of the organism and, hence, also main pathways responsible for drug pharmacokinetics. P-glycoprotein (P-gp) is a drug transporter that plays a pivotal role in drug absorption, distribution, and elimination, and temporal changes in its activity may affect input, output, activity, and toxicity profile of drugs. In the current study, the influence of different circadian stages on the overall intestinal permeability (P_{eff}) of the P-gp substrates talinolol and losartan was evaluated in in situ intestinal perfusion studies in rats. Additionally, in vivo studies in rats were performed by employing the P-gp probe talinolol during the day (nonactive) and night (active) period in rats. Effective intestinal permeabilities of talinolol and losartan were smaller in studies performed during the night ($p < .05$), indicating that P-gp–dependent intestinal secretion is greater during the nighttime activity span than daytime rest span of the animals. P-gp modulators vinblastine and PSC833 led to a significant decrease of talinolol and losartan exsorption in the intestinal segments as compared with control groups. Strikingly, the permeability-enhancing effect of vinblastine and PSC833 was higher with night perfusions, for both talinolol and losartan. In vivo studies performed with talinolol revealed—consistent with the in situ studies (P_{eff} day > night)—a day vs. night difference in the oral availability of talinolol in the group of male rats in terms of the area under the curve (AUC) data ($AUC_{day} > AUC_{night}$). The P-gp modulator vinblastine significantly increased talinolol AUC_{day} ($p < .05$), whereas only a weak vinblastine effect was seen in night. According to the in situ data, the functional activity of P-gp was regulated by the CTS in jejunum and ileum, which are major intestinal segments for energy-dependent efflux. In conclusion, circadian rhythms may affect carrier-mediated active efflux and play a role in the absorption process. In addition to daily rhythms in P-gp activity in rat intestine, the in vivo studies indicate that absorption-, distribution-, metabolism-, and elimination-relevant rhythms may be involved in the circadian kinetics of the drug, besides transporter-dependent efflux, such well-known aspects as metabolic or renal clearance or motility. Since this also holds true for a potentially interacting second compound (modulator), modulator effects should be evaluated carefully in transporter related drug-drug interactions. (Author correspondence: aokyar@istanbul.edu.tr)

Keywords: Chronopharmacokinetics, Circadian rhythm, Intestinal perfusion, Losartan, P-glycoprotein, Talinolol

INTRODUCTION

Time is an essential dimension of all life processes and plays a key role in physiological function of organisms. Most biological functions display rhythmic variations along a wide range of periods. All biological rhythms are governed by the circadian timing system (CTS) in the brain that coordinates physiology and cellular functions, and this implies that the effects of medications may depend on it to some extent. The CTS also controls main pathways responsible for drug pharmacokinetics and modifies absorption, distribution, metabolism, and elimination (and toxicity)—ADME(T)—of drugs during

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the 24 h. It is well documented that the pharmacokinetic profiles of many drugs may vary depending upon their administration time, and circadian changes in metabolic activities appear to be its major cause (Bruguerolle, 2008; Lemmer, 1999; Lévi et al., 2000, 2010). Studies on rats and mice involving drug metabolism revealed a higher metabolic activity during the night period (Belanger et al., 1997; Furukawa et al., 1999; Gachon et al., 2006; Martin et al., 2003). A wide variety of compounds, however, are not only metabolized, but also transported via inside- and/or outside-directed transport systems, e.g., for exsorptive transport through adenosine triphosphate (ATP)-binding cassette (ABC) transporters in particular. P-glycoprotein (P-gp), a member of the ABCB subfamily, is also known as ABCB1 (human) and abcb1a/1b and abcb4 (rodent), and is the most outstanding one among ABC transporters, as it confers the strongest resistance to the variety of antineoplastic compounds (Szakacs et al., 2006). Furthermore, P-gp is expressed at the apical side, i.e., mucosa, of the intestinal layer and is responsible for intestinal secretion of various compounds (Klaassen & Aleksunes, 2010).

Recent studies at the cellular level provide evidence that gene expression of exsorptive transporters is also clock-controlled (Claudel et al., 2007; Gachon et al., 2006; Lévi & Schibler 2007; Lévi et al., 2010; Murakami et al., 2008). The molecular clocks in the liver and intestine control circadian expression of PAR-domain basic leucine zipper (PAR bZip) transcription factors, which, in turn, govern the expression of constitutive androstan receptor (CAR), peroxisome proliferator activated receptor-alpha (PPAR-α), and aryl hydrocarbon receptor (Ahr). CAR, PPAR-α, and Ahr represent nuclear proteins that are responsible for modulating the rhythmic expression of ABC transporters, including P-gp, multidrug resistance–associated protein-2 (MRP2/mrp2/abcc2) and breast cancer resistance protein (BCRP/bcrp1/abcg2) in peripheral organs. Further, it was recently shown that mRNA expression of P-gp in mice shows a daily rhythm in jejunum, ileum, and colon segments of the intestine as well as in the liver and kidney (Ando et al., 2005; Okyar et al., 2009). No circadian changes in the functional activity of P-gp in the intestinal segments have been demonstrated thus far, although preliminary functional data provide evidence for its consistent variations (Lemmer et al., 2004).

In order to determine its relevance in ADME as well as functional test systems for P-gp, suitable probes are needed. Talinolol (TAL) is a β-adrenoceptor antagonist and P-gp substrate and, hence, undergoes P-gp–mediated exsorption in the intestine (Spahn-Langguth et al., 1998; Wetterich et al., 1996). Its metabolic degradation appears to be largely negligible in rats and humans and, hence, a saturable first-pass effect does not play a role. In the liver, talinolol converts into four metabolites in man: 4-*trans*-hydroxytalinolol, 2-*trans*-hydroxytalinolol, 3-*cis*-hydroxytalinolol, and 3-*trans*-hydroxytalinolol. The major metabolite is 4-*trans*-hydroxytalinolol. Less than 5% of talinolol is metabolized in the liver and intestine by cytochrome P450 enzymes (CYPs) (Oertel et al., 1994; Trausch et al., 1995). TAL represents a biopharmaceutical classification system (BCS) class II compound (Amidon et al., 1995; Wu & Benet, 2005). Losartan (LOS) is a highly selective angiotensin II type 1 receptor antagonist, which shows high intestinal permeability. LOS is a P-gp and a CYP3A4 substrate, and CYP2C9, CYP2C10, and CYP1A2 are also involved in its metabolism. It represents a BCS class III compound in humans (Bramlage & Schindler, 2010; Takagi et al., 2006). Because of its advantageous pharmacokinetic properties, TAL was proposed as a suitable model compound for P-gp–mediated drug-drug and drug-food interactions (Spahn-Langguth, 1998; Spahn-Langguth & Langguth, 2001) and is widely used as functional P-gp probe, e.g., for testing the inhibitory effect of herbal preparations on its exsorptive transport (e.g., Fan et al., 2009).

In situ intestinal perfusion represents a frequently used functional model to evaluate the permeability characteristics of drugs (Doluisio, 1969; Fagerholm, 1996; Sandström & Lennernas, 1999). Hanafy and coworkers (2001) previously characterized the overall intestinal permeability (P_{eff}) of TAL in rats and concluded it is a suitable model compound to mechanistically study P-gp–related processes. The purpose of the present study was to assess the day-vs. night- (i.e., rest-vs. activity-period) differences of the effective intestinal permeabilities in rats for the model compounds TAL and LOS (Hanafy et al., 2001; Spahn-Langguth et al., 1998) and to investigate the potential circadian effect of the P-gp modulator vinblastine (VBL) and PSC 833 (PSC) in the two-step perfusion model. Furthermore, the relevance of the in situ results for in vivo conditions is evaluated for a selected setup, where TAL pharmacokinetics in rats is studied.

MATERIALS AND METHODS

Animals and Synchronization

All experiments involved rats that were housed under a 12/12 light/dark cycle. Light was provided by fluorescent lamps and its intensity at cage level ranged from 100 to 150 lux, depending on the cage position in the room. The animal rooms were equipped with temperature control (22°C ± 1°C). Animals were kept in cages housing up to 4 animals. Treatment and sampling of rats during darkness was performed under dim-red light (7 lux).

Preclinical study approvals were obtained from the Committee for Animal Studies of the Martin-Luther-University Halle-Wittenberg for in situ studies and Istanbul University Ethics Committee of Experimental Animals for in vivo investigations. All procedures respected the ethical standards of the journal (Portaluppi et al., 2010). For the perfusion studies, male white Wistar rats were purchased from Charles River (Sulzfeld, Germany). For the in vivo studies, male rats were obtained from the animal facilities of the Istanbul University, Institute of

Experimental Medicine and purchased from Harlan (Blackthorn, UK).

Compounds and Reagents

Racemic TAL was kindly provided for H.S.-L. by AWD Pharma (Arzneimittelwerk Dresden, Radebeul, Germany), and VBL was purchased from GRY-Pharma (Kirchzarten, Germany). PSC (6-[(2*S*,4*R*,6*E*)-4-methyl-2 (methylamino)-3-oxo-6-octenoic acid]-7-L-valine-cyclosporin A) is an investigational compound provided by Novartis (Basel, Switzerland). LOS was obtained from Du Pont Merck (Merck Pharmaceuticals, Wilmington, DE, USA), pindolol from Sandoz (Basel, Switzerland), and benoxaprofen recrystallized and purified from a batch initially obtained through Lilly Research (Windestam, UK). Buffer salts, solvents, and reagents were obtained from E. Merck (Darmstadt, Germany), unless stated otherwise.

In Situ Studies

The study was designed to define the intestinal permeabilities of the two compounds dependent upon the time of their perfusion (daytime rest span vs. nighttime activity span) in a two-step, in situ perfusion model in rats. In situ perfusions (ileum and jejunum) were performed at times defined in terms of hours after light onset (HALO), i.e., from HALO 1 to HALO3 (day/rest span) and also from HALO13 to HALO15 (night/activity span).

The surgical procedure was performed according to the method published by Doluisio et al. (1969). Male Wistar rats (total number 12, n = 6 for each compound/inhibitor, body weights 280–320 g) were fasted for 18 h prior to surgery, but they had unlimited access to tap water. Rats were anesthesized via intraperitoneal (i.p.) administration of ketamine (Ketamin-Ratiopharm, Ulm, Germany; 40 mg/kg) and xylazine (Rompun; BayerVital, Leverkusen, Germany; 10 mg/kg). The anesthetized rats were placed on a 37°C heating pad to maintain body temperature. Intestinal segments of the rats were exposed by a midline intestinal incision and the selected gut segments rinsed and cleaned with Tyrode buffer (pH 7; 37°C) to prevent extensive mucus secretion into the eluate during perfusion, and thereafter silicon tubes were attached to the selected intestinal segments of jejunum (5–8 cm) and ileum (3–7 cm) tissue.

Tyrode solutions were used as perfusion media as described previously (Hanafy et al., 2001). The perfusate (drug-containing Tyrode buffer) was maintained at 37°C by a water bath and delivered through the intestinal segments simultaneously at a constant flow rate of .2 mL/min. At the outlet, perfusate was quantitatively collected at intervals of 5 min and stored at –20°C until chromatography. Adsorption by the plastic silicon tubes does not occur, as shown by blind perfusions (without gut segments), where the respective P_{eff} value was 0 for both talinolol and losartan. A stepwise perfusion was performed, with the total perfusion time of 90 min divided into two periods, each consisting of a 15-min period without monitoring to reach steady-state conditions and a 30-min period for TAL P_{eff} determination at intervals of 5 min (Hanafy et al., 2001). The optimized TAL inhibition model was employed that permits intraindividual comparisons without and with the transport inhibitors/modulators VBL and PSC, respectively. Perfusions were performed in two steps in each animal with TAL (10 μM) and LOS (10 μM) in the absence and presence of VBL (100 μM) and PSC (20 μM), respectively.

Calculation of Water Transport and Calculation of Permeability Coefficients

Water transport was quantified by weight and volume measurements. Usually, water transport was in the range of 5–10%. Intestinal permeabilities were calculated on the basis of the mixing tank model, using the following equation that was initially described by Sinko et al. (1991).

$$P_{\text{eff}} = \frac{\upsilon \cdot \left(\frac{c_{in} - c_{\text{out}}}{c_{\text{out}}} \right)}{2\pi r l}$$

where υ is the flow rate, C_{in} and C_{out} the respective inlet and outlet concentrations of the drug-containing perfusion solution, and r and l the radius and length of the corresponding intestinal segment (average radius of the intestinal segments for jejunum and ileum was determined as .21 cm), respectively.

In Vivo Studies

Male rats (180–200 g, n = 4/sampling point) were studied. The daytime experiment commenced at HALO1 and nighttime experiments at HALO13. Animals were fasted 12 h before experiments and 20 mg/kg TAL was administered orally, alone (control group), or combined with P-gp inhibitor VBL (.1 mg/kg, i.v). Blood was sampled from the heart (4–5 mL) 2, 4, 6, and 8 h after dosing, and plasma was separated and stored at –25°C until analysis. TAL determination in plasma samples was performed using the validated high-performance liquid chromatography (HPLC) method described below (Okyar, 2004).

Bioanalytical Assays

TAL Determination From Plasma

A method modified from Oertel et al. (1994; Okyar, 2004) was used to determine TAL from rat plasma. TAL was extracted from plasma via hydrophilic-lipophilic balance type (HLB) solid-phase extraction cartridges (Oasis SPE cartridges 1 cc/30 mg; Waters, Milford, MA, USA) and analyzed by reversed-phase Symmetry C_{18} 250 × 4 mm column (Waters) connected with Symmetry Sentry (2.1 × 3.9 mm; 5 μm) precolumn (Waters). The extraction was carried out on a VacElut (Varian, Palo Alto CA, USA) sample preparation system with a vacuum pump (KNF, Freiburg, Germany). Briefly, cartridges were conditioned with 1 mL methanol and

HPLC-grade water; then, 1 mL plasma plasma was passed through the cartridges. Cartridges were rinsed with 1 mL water, then water:methanol (95:5, *v*/*v*) for unretained matrix, and, finally, TAL was collected in clean tubes by eluting it with 1 mL pure methanol. Analyses were performed on an HPLC system consisting of a Waters 2695 pump, autosampler, column heater, and Waters 2487 ultraviolet/visible (UV/VIS) detector at a wavelength of 242 nm for eluate monitoring (Waters). A mixture of .05 M phosphate buffer/acetonitrile (73:27, *v*/*v*; pH 4) was used as a mobile phase for HPLC; flow rate was 1 mL/min, and the column temperature was adjusted to 40°C. Retention time of TAL was 9.5 min, and the limit of determination was 50 ng/mL. The calibration curve was linear within the range of 50–2000 ng/mL. Variability of the assay was in the range of 3–7%, and no interfering peak was detected during the analysis that was related to VBL.

TAL Determination From Buffer

TAL concentration in the perfusate was enantioselectively determined using an HPLC-UV/VIS detector as previously described (Wetterich et al., 1996). From perfusate-sample aliquots (100 µL), TAL enantiomers were separated by liquid-liquid extraction (dichloromethane/isopropanol, 95:5, *v*/*v*; 2 mL) following addition of internal standard rac-pindolol and .1 mL 1 M sodium hydroxide. The organic layer was collected in a tube and evaporated under a stream of nitrogen at 40°C. The samples were reconstituted in methanol and analyzed by HPLC on a ChiraSpher NT 5 µm 250 × 4mm column (Merck, Darmstadt, Germany) connected with LiChroCART 4-4 LiChrospher C_{18} (4 × 4 mm; 5 µm) precolumn (Merck). Ethanol triethylamine (1000:.05, *v*/*v*) was used as mobile phase with a flow rate of .45 mL/min; column temperature was room temperature (20°C) and the wavelength for eluate monitoring was 245 nm. Average retention times were as follows: S-TAL was 15.5, R-TAL was 17.9, S-pindolol was 19.6, and R-pindolol was 21.1 min. The calibration curve ranged from 200 to 10 000 ng/mL for each enantiomer. Variability of the assay was in the range of 2–10%, and the limit of determination was 10 ng/mL per enantiomer. Also, here, neither VBL nor PSC showed interference with the assay.

LOS Determination From Buffer

LOS was extracted from buffer solution via liquid-liquid extraction, and its concentration was determined by HPLC-fluorescence detection (excitation 280 nm/emission 380 nm) as described by Dressler (2002). Briefly, 2 mL methyl-*tert*-butylether (MTBE) and 100 µL internal standard solution (benoxaprofen, 250 ng/mL) were added to 350 µL buffer and the mixture acidified with 1.4 mL 2 M HCl. The acidic mixture was shaken horizontally for 30 min and centrifuged for 15 min at 4000 rpm. The organic layer was transferred into a second tube, acidified with .5 mL .1 M HCl, and then shaken again for 30 min. In the last step, the collected organic layer was evaporated under a stream of nitrogen gas at 40°C. The residue was reconstituted in 100 µL mobile phase, and 75 µL was injected onto the system. The analysis was performed by HPLC with Zorbax CN 5 µm 150 × 4 mm column (Bischoff, Leonberg, Germany) connected with a Phenosphere CN 60 × 4 mm precolumn (Phenomenex, Aschaffenburg, Germany). A mixture of 5 mM sodium dihydrogen phosphate:acetonitrile:methanol:tetrahydrofuran (73:17.5:8.0:4.0, *v*/*v*) was used as mobile phase (pH: 3.15), at a flow rate of 1.3 mL/min, with the column temperature at room temperature (20°C). Benoxaprofen was employed as internal standard. Retention time of LOS was 8.8 min, and the limit of determination was 10 ng/mL. The calibration curve, ranging from 40 to 4000 ng/mL, was linear. Variability of the assay was in the range of .55–5.81%, and neither VBL nor PSC interfered with the analysis.

Kinetic and Statistical Analyses

Averaged concentrations for each time point were used to calculate the area under the curve (AUC) from $t = 0$ to the last sampling point (AUC_{0-8h}) by the linear trapezoidal rule; standard errors (SEM) were calculated by the law of propagation of errors (Bardelmeijer et al., 2000). The maximum plasma concentrations (C_{max}) were directly observed from the plasma concentration–time curves and expressed as mean ± standard error of mean (SEM). Other talinolol pharmacokinetic parameters were calculated by noncompartmental analysis (WinNonlin Phoenix 6.0; Pharsight, Mountain View, CA, USA) and expressed as mean and standard error of mean. Terminal phase half-life ($t_{1/2}$) was approximated by linear regression analysis from the ln*C*-values of the terminal phase of the overall profile. Half-life and terminal rate constant (k_{el}) were interconverted as follows:

$$t_{1/2} = \ln 2 / k_{el}$$

The apparent total clearance after per oral dosage (CL/*F*) was calculated as dose/AUC_{total}, and the apparent volume of distribution (V_d) as CL/k_{el}. In situ and in vivo data were analyzed for statistical significance of differences between groups by the nonparametric Mann-Whitney test (Sachs, 1978).

Prediction of P_{eff} via Gastroplus/ADMET Predictor

Log*P* as well as P_{eff} values for rat, mouse, and man were estimated via the GastroPlus program (version 6.0) from SimulationPlus (Lancaster, CA, USA) to be compared with experimental data from the current study.

RESULTS

In Situ Studies

TAL P_{eff} Differences Between Day (Rest) and Night (Activity) Periods

Effective intestinal permeabilities of TAL in rats were lower when the studies were performed during the

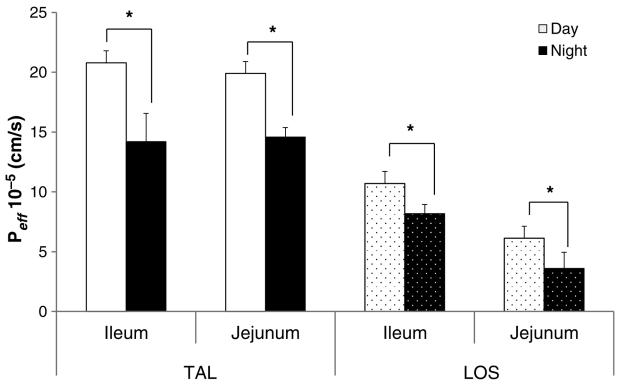

FIGURE 1. TAL and LOS intestinal perfusion studies: day vs. night. Comparison of P_{eff} values (10^{-5} cm/s) of TAL and LOS obtained during the daytime and nighttime experiments (means ± SEM [n = 6]; *$p < .05$). Lower permeabilities may be a result of higher P-gp activity.

night. For TAL, P_{eff} values were higher during the daytime rest period of the rats. Nighttime perfusions performed in the ileum yielded significantly smaller P_{eff} values ($p < .05$), calculated as 20.8 (± 1.64) × 10^{-5} and 14.2 (± 2.36) × 10^{-5} cm/s for the day (rest) and night (activity) spans, respectively, indicating a higher P-gp activity during the nighttime activity period (Figure 1). Nighttime perfusions performed in the jejunum also displayed significantly smaller P_{eff} values ($p < .05$) and were calculated as 19.9 (± .93) × 10^{-5} and 14.6 (± .79) × 10^{-5} cm/s for the day and night spans, respectively (Figure 1).

The obtained data also show the magnitude of the P_{eff}-enhancing effect of VBL and PSC (presumably mainly through P-gp inhibition) was higher for nighttime perfusions. When VBL was added to the perfusion solution, effective permeability was doubled in both the jejunum and ileum as compared to control experiments. Ileum perfusions displayed 83.7% enhancement during the daytime and 124.7% increment during the nighttime. Similarly, jejunum perfusions performed with the addition of VBL yielded 105.5% and 122.6% enhancement of P_{eff} for the day and night studies, respectively.

When PSC was added to the perfusion solution, P_{eff} was increased in both the ileum and jejunum relative to the control. Ileum perfusions showed 30.8% enhancement during daytime and 71.8% increment during nighttime studies. Similarly, jejunum perfusions performed with PSC provided 43.2% and 63.7% enhancement of P_{eff} for the day and night studies, respectively (see Figure 2a).

LOS P_{eff} Differences Between the Day and Night Periods

Also for LOS, effective intestinal permeabilities were significantly lower ($p < .05$) when the perfusion studies were performed during the night than day. LOS P_{eff} values were higher during the daytime rest period. Nighttime perfusions performed on the ileum provided smaller P_{eff} value, and this effect was statistically significant ($p < .05$). The P_{eff} value amounted to 10.7 (± .50) × 10^{-5} and 8.18 (± .75) × 10^{-5} cm/s for the day and night

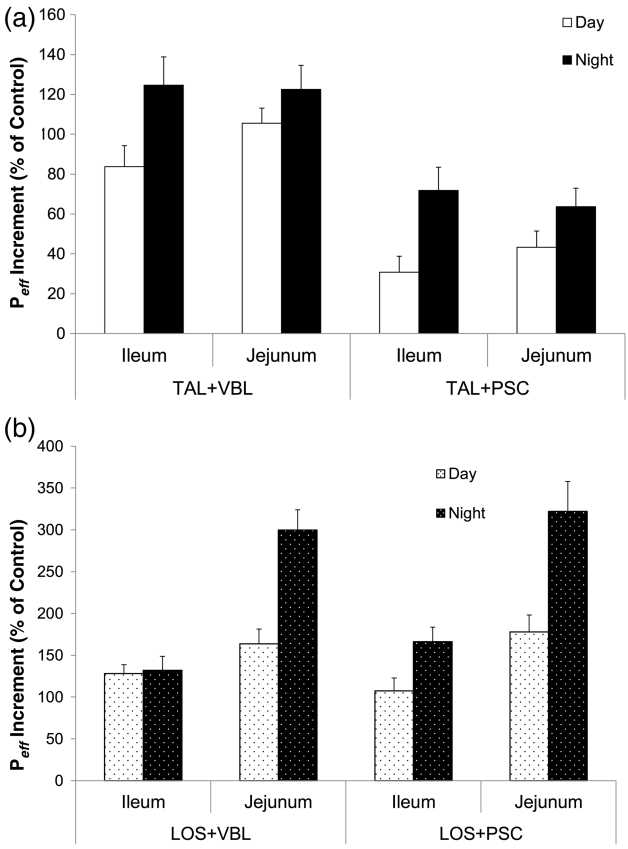

FIGURE 2. (a) TAL perfusions with P-gp inhibitors. Percent increase of P_{eff} (% of control, see Figure 1) of TAL upon addition of inhibitors to buffer solution of VBL and PSC. The higher increase of P_{eff} upon addition of modulators/inhibitors may also be explained by a higher contribution of inhibitable processes during the activity (night) period (n = 6). (b) LOS perfusions with P-gp inhibitors. Percent increase of P_{eff} (% of control) of LOS upon addition of inhibitors to buffer solution of VBL and PSC (n = 6).

studies, respectively (Figure 1). Night perfusions performed in the jejunum also displayed significantly smaller P_{eff} values ($p < .05$) as compared to day experiments and were calculated to be 6.13 (± 1.04) × 10^{-5} and 3.60 (± 1.35) × 10^{-5} cm/s for the daytime and nighttime assessments, respectively (Figure 1).

According to the data obtained, the P_{eff}-enhancing effect of VBL and PSC for LOS was higher during the nighttime activity span. Ileum perfusions with VBL provided a 128% increase in daytime (rest span) studies and a 132% increment for the nighttime (activity span) studies, the respective values for jejunal perfusions were 164% and 300% increase for day and night, respectively. A highly significant increase of LOS permeability of ≈300% was detected in jejunal coperfusions with PSC as inhibitor during the nighttime (see Figure 2b).

Comparison of In Situ and In Silico Permeabilities for TAL and LOS

Predicted effective permeabilities (as estimated via Gastroplus) and P_{eff} values obtained as averages of six rats in the experiments performed in the absence and

TABLE 1. Experimentally estimated (day and night) and predicted effective intestinal permeabilities for TAL and LOS in rats as well as relative changes upon inhibitor addition (average change) and permeability ratios with/without inhibitor for each studied daytime period

(a) Talinolol

Chronodependence of basic parameter	Jejunum	Ileum
In silico rat P_{eff} ($\times 10^{-5}$ cm/s)	16.8	16.8
Measured rat P_{eff} (day) ($\times 10^{-5}$ cm/s) = $P_{eff\text{-}0}$	19.9	20.8
Measured rat P_{eff} (night) ($\times 10^{-5}$ cm/s) = $P_{eff\text{-}0}$	14.6	14.2
Ratio $P_{eff\text{-}0}$ (day)/$P_{eff\text{-}0}$ (night) ($\times 10^{-5}$ cm/s)	1.36	1.46
Influence of modulators: Values as ratios of respective P_{eff}s (without vs. with modulator; i.e., values >1 indicate increase; $P_{eff\text{-}0}$ = control) and increment by modulator (in parentheses).		
$P_{eff\text{-}0}/P_{eff\text{-}VBL}$ (day)	.49 (+106%)	.54 (+84%)
$P_{eff\text{-}0}/P_{eff\text{-}PSC}$ (day)	.44 (+43%)	.44 (+31%)
$P_{eff\text{-}0}/P_{eff\text{-}VBL}$ (night)	.70 (+123%)	.76 (+125%)
$P_{eff\text{-}0}/P_{eff\text{-}PSC}$ (night)	.61 (+64%)	.58 (+72%)

(b) Losartan

Chronodependence of basic parameter	Jejunum	Ileum
In silico rat P_{eff} ($\times 10^{-5}$ cm/s)	2.3	2.3
Measured rat P_{eff} (day) ($\times 10^{-5}$ cm/s) = $P_{eff\text{-}0}$	6.1	10.7
Measured rat P_{eff} (night) ($\times 10^{-5}$ cm/s)= $P_{eff\text{-}0}$	3.6	8.2
Ratio P_{eff} (day)/P_{eff} (night) ($\times 10^{-5}$ cm/s)	1.70	1.31
Influence of modulators: Values as ratios of respective P_{eff}s (without vs. with modulator; i.e., values >1 indicate increase; $P_{eff\text{-}0}$ = control) and increment by modulator (in parentheses).		
$P_{eff\text{-}0}/P_{eff\text{-}VBL}$ (day)	.38 (+164%)	.44 (+128%)
$P_{eff\text{-}0}/P_{eff\text{-}PSC}$ (day)	.25 (+178%)	.43 (+108%)
$P_{eff\text{-}0}/P_{eff\text{-}VBL}$ (night)	.36 (+300%)	.48 (+132%)
$P_{eff\text{-}0}/P_{eff\text{-}PSC}$ (night)	.24 (+322%)	.38 (+166%)

Other reference values: TAL (talinolol): predicted P_{eff} of TAL was determined as 12.9×10^{-5} cm/s using the in situ rat jejunal loop method (Shirasaka et al., 2009). Predicted P_{eff} of TAL was determined as 3.1×10^{-5} cm/s for humans by Gastroplus. LOS (losartan): predicted P_{eff} of LOS was determined as 9.3×10^{-5} cm/s for man via Gastroplus. LOS data, determined by Therapeutic Systems Research Lab. (http://www.tsrlinc.com), was 11.5×10^{-5} cm/s. With respect to the permeability of the modulator VBL, in silico P_{eff} values were calculated as 2.0×10^{-5} cm/s for human and 5.7×10^{-5} cm/s for rat by Gastroplus.

presence of modulators is shown in Table 1a and b. The in silico effective permeability value of TAL was calculated to be 16.8×10^{-5} cm/s in rats. In the current rat studies, this value was estimated under the various conditions to be as follows: P_{eff} (day) 19.9×10^{-5} vs. P_{eff} (night) 14.6×10^{-5} cm/s for TAL in the jejunum, whereas in ileum the respective values were 20.8×10^{-5} (day) vs. 14.2×10^{-5} (night) cm/s.

For LOS, the in silico effective permeability was calculated as 2.3×10^{-5} cm/s in rat using GastroPlus. In the rat study, P_{eff} was determined as 6.13×10^{-5} vs. 3.6×10^{-5} cm/s in the jejunum during day vs. night, respectively, and in rat ileum as 10.7×10^{-5} vs. 8.2×10^{-5} cm/s during day vs. night, respectively.

In Vivo Studies

Plasma concentration–time profiles after oral administration of TAL with and without VBL coadministration are shown in Figure 3a and b, respectively. The plasma concentration reached its maximum within the first 2 h in both day and night studies, i.e., no t_{max} difference was detected. It was observed that for orally administered TAL with and without VBL that the absorption phase was largely completed after 2 h. Upon VBL coadministration, a 119% enhancement of TAL C_{max} was found during the daytime, which was significantly different from the respective daytime control group ($p < .05$). Yet, no significant difference was found for C_{max} values of TAL when comparing control and VBL groups in nighttime experiments, showing a negligible/inferior effect of VBL coadministration during the night. Other pharmacokinetic parameters calculated after oral administration of TAL are shown in Table 2.

In accordance with the results from in situ studies, where a lower intestinal permeability was detected during the nighttime, TAL $AUC_{0\text{–}8h}$ values in daytime experiments ($1.53 \pm .43$ μg·h/mL) were 21% higher than in the nighttime experiments ($1.26 \pm .23$ μg·h/mL). VBL coadministration increased TAL AUC significantly ($p < .05$) during the day ($2.56 \pm .62$ μg·h/mL), but only slightly on average and not significantly during the night ($1.38 \pm .22$ μg·h/mL), as shown in Figure 4. The VBL-associated relative enhancement of the TAL AUCs amounted to 67.3% during the day and showed only a slight increase of 10% at night.

DISCUSSION

Time of administration has a prominent role on absorption, distribution, metabolism and elimination of medications. Since approximately 75–80% of drugs are

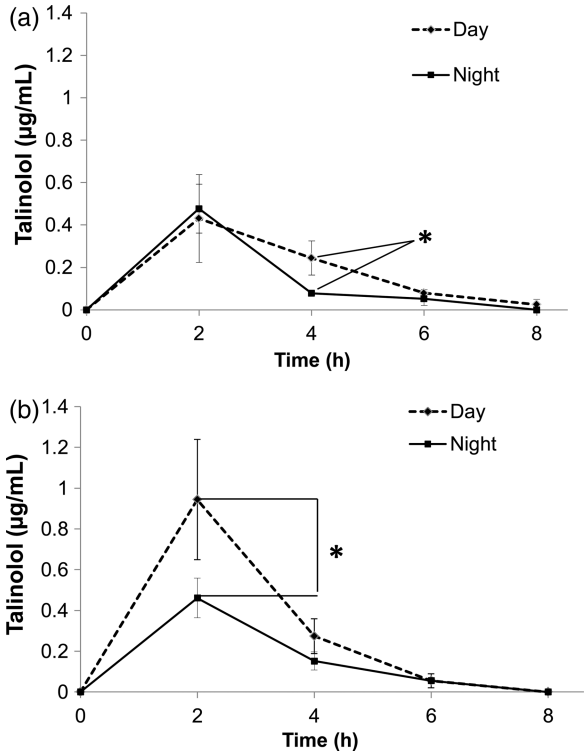

FIGURE 3. Plasma concentration-time profiles after oral administration of TAL alone (a) and upon VBL (b) coadministration to male Wistar rats in daytime and nighttime studies. Data are summarized as means ± SEM (n = 4). Mean TAL plasma level at 4 h was higher after daytime than nighttime drug administration (Mann-Whitney, $^*p < .05$). VBL coadministration caused statistically significant enhancement of TAL plasma concentration at 2 h during daytime as compare to nighttime (Mann-Whitney, $^*p < .05$).

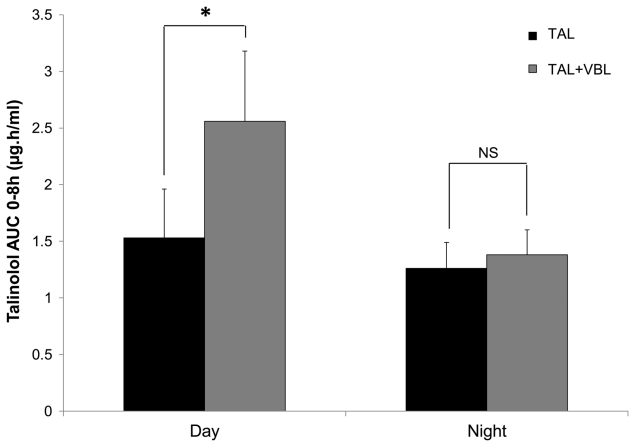

FIGURE 4. Average AUC values in µg·h/mL after oral administration of TAL to male Wistar rats without and with VBL coadministration in daytime and nighttime studies (meas ± SEM [n = 4]). VBL coadministration increased significantly TAL AUC during the day (Mann-Whitney, $^*p < .05$). No statistically significant increment was found in TAL AUC when VBL was coadministered at night.

applied via the oral route, temporal variations in the absorption process, e.g., due to rhythms in gastrointestinal motility (Goo et el., 1987) or blood perfusion (Lemmer & Nold, 1991), may considerably affect drug pharmacokinetics and therapeutic outcome (see Lemmer & Bruguerolle, 1994; Lemmer et al., 1991).

Circadian clocks control the transcription of some ABC family members, including mRNA of P-gp (abcb1a/1b) in mouse liver (Zhang et al., 2009) and intestine (Ando et al., 2005; Murakami et al., 2008; Okyar et al., 2009). P-gp acts as an intestinal barrier to limit oral drug absorption. Thus, alteration of the efflux function of this carrier may be responsible for variation in drug pharmacokinetics. P-gp is highly expressed in rat ileal and jejunal mucosa, and its abundance increases from the proximal to distal regions of the intestine, with no sex-specific differences observed (MacLean et al., 2008). We hypothesized that daily variations of P-gp function in the intestine would affect the pharmacokinetics of P-gp substrates. Administration-time-related differences were also shown in the current study, where effective intestinal permeabilities of TAL and LOS were studied via in situ intestinal perfusion, and, additionally, in vivo studies in rats were performed to explore the relevance of circadian changes of the intestinal secretion process with respect to drug absorption and bioavailability.

The studies were performed in rats, and this gives an additional aspect to the relevance of the study: preclinical kinetic and dynamic studies are frequently performed in rats; however, it is usually neglected that rats, as are other rodents, night-active as opposed to humans, who are diurnally active. One may argue this lacks relevance for

TABLE 2. Pharmacokinetic parameters calculated after oral administration of TAL (20 mg/kg) to male Wistar rats in the absence and presence of VBL at HALO1 or HALO13

PK Parameters	TAL (HALO1)	TAL + VBL (HALO1)	TAL (HALO13)	TAL + VBL (HALO13)
AUC_{0-8h} (µg·h/mL)	1.53 ± .43	2.56 ± .62*	1.26 ± .43	1.38 ± .22
C_{max} (µg/mL)	.43 ± .20	.94 ± .29#	.47 ± .12	.46 ± .10
k_{el} (h^{-1})	.42	.64	.55	.73
$t_{1/2}$ (h)	1.63	1.10	1.25	1.00
V_d/F (L)	6.21	2.43	5.76	3.95
CL/F (L/h)	2.51	1.56	3.17	2.89

Data are expressed as means ± SEM for AUC and C_{max} and mean for other pharmacokinetic parameters. VBL coadministration caused statistically significant enhancement of the AUC of TAL (Mann-Whitney, $^*p < .05$) and of the plasma concentration at 2 h (C_{max}) during daytime (Mann-Whitney, $^{\#}p < .05$).

in situ perfusions or studies with excised tissues, but metabolic activity is known to be clock-controlled within each cell, and this is known to have impact on P_{eff} values of compounds with a considerable metabolic clearance fraction and lead to administration-time differences in kinetics. Yet, the focus of the current studies is mainly oral availability and exsorptive transport.

In Situ Studies: Day vs. Night Comparison, Including VBL and PSC Effects on TAL P_{eff} and VBL and PSC Effects on LOS P_{eff}

From the current studies, it is concluded that P-gp activity is greatest at night, which is in agreement with findings from P-gp (abcb1a/1b) mRNA-expression studies in male C57/BL6J mice jejunum and male $B6D2F_1$ ileum and colon mucosa (Ando et al., 2005; Okyar et al., 2009). As expected, VBL and PSC inhibit the exsorption process in both day and night experiments and cause increase in P_{eff} values. Strikingly, the magnitude of the TAL-P_{eff}-enhancing effect of VBL and PSC was higher for nighttime (activity span) perfusions. When interpreting these data, it needs to be assumed that TAL clearance is mainly nonmetabolic, which was proven by various investigators, although a very minor metabolic clearance was detected as well (Trausch et al., 1995; Zschiesche et al., 2002). Nevertheless, for the current drug-drug interaction study with TAL, metabolic clearance may play a role as well, yet only for the two potential P-gp modulators, which were added to the perfusates. However, no data on VBL or PSC were collected in the course of the present study.

The VBL perfusate levels appear largely constant as assumed for single-pass perfusion, suggesting that VBL absorption is strongly restricted by P-gp during the nighttime activity span, especially in the jejunal region of the small intestine. Yet, Nakayama and coworkers (2000) stated that VBL disappeared from duodenal and ileal loops of male Wistar rats fairly rapidly (30–60% in 30 min). This would not only cause rapid decrease in jejunal levels, but also significant levels at the major LOS and VBL clearance site, i.e., intestinal and hepatic metabolism through CYP isoforms, with the potential of a deviating outcome with deviation in either VBL or LOS first-pass or total blood clearance. The magnitude of the LOS-P_{eff}-enhancing effect of VBL and PSC was greater with nighttime than daytime perfusions. LOS is subject to considerable intestinal metabolism via CYP3A, in addition to intestinal excretion via P-gp. In the present studies, it was observed that VBL and PSC inhibit intestinal secretion of LOS, but this effect was remarkable during the nighttime activity period, only. It is probable that VBL (and also PSC to some extent) not only inhibited carrier-mediated intestinal secretion, but also CYP3A-dependent intestinal metabolism of the tested drugs, particularly LOS. This conclusion is based on published data showing that VBL and to some minor extent also PSC inhibit CYP3A activity (Achira et al., 1999; Fischer et al., 1998). In summary, the transport inhibition experiments indicate that permeability enhancement is largely related to efflux reduction. However, in general, also intestinal metabolism appears to be of relevance for the explanation of the daytime vs. nighttime differences.

In Vivo Talinolol Studies for Day vs. Night Comparison

To verify the relevance of the time-dependent changes of P-gp activity in vivo, further investigations included TAL pharmacokinetics in (male) rats at HALO1 and HALO13. The detected difference was consistent with the in situ studies (P_{eff} day > night), i.e., a day-vs. night-difference in the oral bioavailability of TAL was detected in vivo in male rats. Yet, that AUC was slightly higher at HALO1 as compared to HALO13 (~10% higher, n.s.) may be explained by increased jejunal and ileal blood flow during the nocturnal activity span in rodents. On the other hand, C_{max} values were almost the same for the two times of day (rest span .43 μg/mL vs. activity span .47 μg/mL). In rodents, motor activity and gastrointestinal motility are higher during the activity span (Lemmer, 1981). Apparently, during the night span, higher P-gp activity restricted TAL absorption, but the increment in intestinal blood flow and motility might partially or totally augment TAL uptake in the intestine. If only fluctuations in P-gp had played a role in TAL secretion, most probably one would have seen a smaller TAL AUC at night. As opposed to the TAL in vivo findings, in a previous study with digoxin, a pure P-gp substrate like TAL in male Wistar rats, digoxin plasma levels and AUC were determined (Hayashi et al., 2010). Strikingly, AUC values of digoxin were higher when the drug was administered at HALO0 (beginning of the rest span) than at HALO12 (beginning of the activity span), although the difference was not statistically significant. Hayashi and coworkers also showed that the AUC of digoxin was higher when the drug was administered at HALO6 than HALO12 (again no statistical significance). In an earlier clinical study, .25 mg digoxin administered orally at 08:00 and 20:00 h to fed male and female healthy volunteers yielded other interesting findings; t_{max} was statistically significantly shorter (54 min) following 08:00 as compared to 20:00 h (96 min) dosing, and C_{max} and AUC values were higher after morning than evening dosing, although the differences were not statistically significant (Erol et al., 2001). It is of interest that in 1988 Bruguerolle et al. showed that t_{max} was shorter and C_{max} higher for oral digoxin in elderly than young patients.

The in vivo picture becomes even more complex when the modulator effect is analyzed. In the daytime experiment, it was observed that upon VBL coadministration the AUC of TAL was increased by 67%, whereas no clear VBL effect was found at night, and the respective calculated increment was only 10%, i.e., much smaller than the influence observed in situ. Increased VBL metabolism at night via CYP3A may be a possible explanation. Temporal variability of most microsomal oxidases occurs with highest—several-fold—activity during the

dark span (activity phase of rats) and, consequently, lowest activity during the rest span, as detected for the liver and intestine of rats and mice (Belanger, 1988; Gachon et al., 2006; Martin et al., 2003). On the other hand, VBL represents not only a CYP3A substrate, but also a substrate for abcb1a/1b and abcc2, which are involved in biliary and intestinal excretion of VBL (Klaassen & Aleksunes, 2010). It was reported that abcc2 mRNA expression is higher during the night than day in male Sprague-Dawley rat jejunum and male $B6D2F_1$ mouse ileum mucosa. The peak mRNA expression was at HALO12 for both tissues (Okyar et al., 2011; Stearns et al., 2007). Moreover, VBL was eliminated mainly by biliary and to some extent renal excretion in rats (Zhou et al., 1990). Basal bile flow is higher during the night than day span in rats (Ho et al., 1975), and glomerular filtration rate (GFR) and renal blood flow are rhythmic, being higher at night (activity span) in rodents (Pons et al., 1994, 1996; Rebuelto et al., 2003). We may claim that VBL elimination is relatively higher in the activity period of the rats. Hence, considering all these pathways, the modulator may exhibit a higher clearance at night. From currently available knowledge, we hypothesized that VBL is more efficiently detoxified during the night span and that its inhibitory effect on P-gp would, hence, be less through its higher clearance and lower blood levels at night.

In conclusion, circadian rhythms may influence the pharmacokinetics of drugs and play a role in the absorption process when affected by P-gp–mediated efflux. However, differences in physiological processes and in the detoxification systems in the organism, in particular, metabolic degradation governed by the CTS, would also contribute to CTS phenomena, as well affect the overall pharmacokinetics of drugs, and might lead to (quantitatively) unexpected outcomes in drug-drug interaction studies. In addition to daily rhythms in P-gp transporting activity in the rat ileum and jejunum, the in vivo studies of our experiments showed that alternative factors might be involved in circadian ADME of the drug besides carrier-mediated efflux. This implies that modulator effects should be evaluated carefully in transporter-related drug-drug interactions. Also, drug-drug interaction studies and their outcome need to be interpreted according to circadian aspects/phenomena, since the outcome of rodent studies may depend on the timing of procedures; thus, knowledge of the time dependence of clearance routes of both drugs is required in order to ensure the predictive value of findings derived from rodent models to humans.

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