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The effects of P-glycoprotein inhibitor zosuquidar on the sex and time-dependent pharmacokinetics of parenterally administered talinolol in mice

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ABSTRACT

P-glycoprotein (P-gp) is an efflux protein that forms a tissue barrier and plays a role in the pharmacokinetics of drugs, limiting the influx of them and other xenobiotics into the cells, as expressed in various tissues such as liver, brain, intestinal mucosa and kidneys. Circadian clock controls many biological functions in mammals including xenobiotic metabolism and detoxification. Circadian rhythms of biological functions may affect the pharmacokinetics, and thus efficacy and/or toxicity of drugs. Aim of this study is to determine how the intraperitoneally administered pharmacokinetics of talinolol, as the probe substrate of P-gp, will change depending on the circadian time and sex in the presence of P-gp inhibitor zosuquidar. 20 mg/kg talinolol with or without 30 mg/kg zosuquidar was administred intraperitoneally to male and female mice at day period (ZT3) and night period (ZT15). Plasma and tissue concentrations of talinolol were determined by using validated HPLC/UV method. The protein levels of P-gp in the liver and small intestine in male and female mice were determined by PCR and Western blot techniques. P-gp protein levels in liver and ileum tissues were not different in female mice but higher in ZT15 as compared to ZT3 in male mice ($p < 0.05$). There was no statistically significant difference in talinolol concentration depending on time and sex in the plasma and liver. There was significant time-dependent difference between ZT3 and ZT15 groups in ileum $AUC_{0-5\ h}$ of talinolol ($p < 0.01$). Talinolol plasma and liver $AUC_{0-5\ h}$ were increased by zosuquidar administration regardless of dosing-time and sex ($p < 0.05$). Our study findings are considerable in terms of revealing changes in pharmacokinetic profiles of P-gp substrates due to the time of administration in combination with P-gp inhibitors/modulators in managing polypharmacy.

1. Introduction

ATP-binding cassette (ABC) transporter P-glycoprotein (P-gp; Multi-Drug Resistance 1; ABCB1, ATP Binding Cassette B1), is a transmembrane protein functioning as an ATP-dependent efflux pump and works as a biological barrier by expelling several drugs/ xenobiotics out of cells in healthy tissues as well as in tumor cells (Lin and Yamazaki, 2003; Stavrovskaya and Stromskaya, 2008). P-gp is expressed in various tissues in mammals including liver, intestinal mucosa and kidney (Lin and Yamazaki, 2003). Its anatomical localization suggests that it plays a pivotal role on drug pharmacokinetics in humans and animals by affecting absorption, distribution, and excretion processes of drugs directly and metabolism indirectly (Cascorbi, 2011; Lin and Yamazaki, 2003; Takano et al., 2006). Inhibition of P-gp in the intestinal mucosa, liver and kidneys may enhance the systemic exposure and decrease the clearance of substrate drugs by diminishing intestinal absorption, increasing biliary excretion and/or renal secretion.

P-gp has a wide variety of substrate specificity including anticancer agents, β-blockers, calcium channel blockers, cardiac glycosides, antibiotics and steroid hormones (Balayssac et al., 2005; Fromm, 2002; Schinkel and Jonker, 2003; Zhou, 2008).Talinolol is a well-known and frequently used probe substrate of P-gp with its advantageous pharmacokinetic properties in order to determine the role of this transporter in drug pharmacokinetics and P-gp-dependent drug-drug interaction studies (Fan et al., 2009; Hanafy et al., 2001; Spahn-Langguth et al., 1998). Talinolol is a long-acting, cardioselective β1-adrenerjic receptor blocker (Trausch et al., 1995; Wetterich et al., 1996). The oral bioavailability of talinolol is 55% and it undergoes active intestinal secretion via P-gp. It is a suitable model compound for the experimental

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studies on P-gp-related processes with the advantages of very low metabolic clearance (<1%; that means negligible metabolism via CYP3A4), broad therapeutic range with well tolerability, weak protein binding (25%) (Oswald et al., 2011; Spahn-Langguth et al., 1998; Trausch et al., 1995; Wetterich et al., 1996).

To better understand the involvement of P-gp on the pharmacokinetics of substrate drugs, P-gp modulators are widely used in experimental studies. The magnitude of P-gp's impact on the intestinal absorption, distribution and elimination processes of substrate drugs become more apparent compared to other biological factors when used with modulators. In clinical studies, P-gp modulators are used to increase drug accumulation in P-gp expressing tumors by reversing multidrug resistance and in normal tissues leading to improvements in therapeutic outcomes in patients (Lin and Yamazaki, 2003; Lund et al., 2017). Zosquidar (LY335979) is a potent (Ki 59 nM) and highly selective modulator of P-gp with IC50 value of 1.2 nM (Dantzig et al., 2001, 1999; Green et al., 2001). It does not inhibit other ABC transporters (MRP1, MRP2 and BCRP) or cytochrome P450 isozymes at concentrations below the micromolar range (Dantzig et al., 1999; Shepard et al., 2003).

P-gp expression indicates sex-specific changes. Studies have shown that the circulating sex hormones regulate P-gp expression and function in systemic organs, causing sex-related differences in drug response (Arceci et al., 1988; Fedoruk et al., 2004; Kim and Benet, 2004; Mutoh et al., 2006; Suzuki et al., 2006).

In mammals, a variety of biological functions are controlled by the Circadian Timing System (CTS) over a 24-h period. Metabolic enzymes and drug transporters in detoxification pathways are under the control of the circadian clock, and they show circadian rhythm at gene expression and protein level (Dibner et al., 2010; Lévi et al., 2010). It is known that the pharmacokinetic profiles of many drugs may vary depending on their administration time, and circadian changes in drug transporters appear to be the major cause (Bruguerolle et al., 2008; Lévi et al., 2010; Levi and Schibler, 2007; Ohdo, 2010, 2007).

The intraperitoneal (i.p.) administration route is one of the more commonly used routes in many in vivo studies of disease models in rodents (Al Shoyaib et al., 2020). On the other hand in clinical practice, some cancers of the abdominal or gastrointestinal region can be treated using an i.p. chemotherapy with cytotoxic drugs which are mostly P-gp substrates (Lagast et al., 2018). There are several studies evaluating the time, sex, and strain-dependent differences in the pharmacokinetics of orally administered P-gp substrates (Ozturk et al., 2017; Okyar et al., 2019; Bicker et al., 2020). However there is only few studies regarding this issue evaluating the role of i.p. route and still remains unclear.

In our study, we aimed to determine the effects of time- and sex-dependent modulation of P-gp via zosuquidar on the systemic exposure of intraperitoneally administered talinolol.

2. Materials and methods

2.1. Animals and synchronization

C57BL/6 J mice in both sexes, 7–8 weeks of age, were purchased from Istanbul University Aziz Sancar Institute of Experimental Medicine. Mice were housed at standard temperature (22 ± 2 °C), illumination (12 h of light/12 h of darkness) and humidity (55 ± 5%). Water and food were provided *ad libitum* throughout the experiments. Mice were synchronized for 3 weeks prior to any intervention with an alternation of 12 h of light and 12 h of darkness (LD12:12) in an autonomous chronobiological facility and the same lighting regimen continued to the end of the experiment. Light intensity at cage level ranged from 220 to 315 lux. All the manipulations during dark span were performed under dim red light (7×10^4 erg/cm^2). The study protocol has been approved by the Istanbul University Local Ethics Committee of Animal Experiments (IUHADYEK, No: 2013/60, 2015/32, 2016/52).

2.2. Drugs and chemical reagents

Talinolol was kindly provided by the AWD (Arzneimittelwerk Dresden, Radebeul, Germany), and zosuquidar was purchased from Medkoo Biosciences (NC, USA). Escitalopram was kindly provided by Neutec Pharmaceutical Co. (Istanbul, Turkey) and was used as an internal standard (IS). All other compounds, solvents and reagents were purchased from E. Merck (Darmstadt, Germany) unless stated otherwise. HPLC grade water was obtained from Elga Purelab Option Q (High Wycombe, UK).

2.3. Pharmacokinetic study design

P-gp substrate talinolol at 20 mg/kg dose and/or zosuquidar (specific P-gp inhibitor) at 30 mg/kg dose were administrated i.p. to female and male mice. Talinolol administration was performed either at the 3rd hour following the light onset (ZT3; Zeitgeber Time 3) or at the 3rd hour following the dark onset (ZT15). Zosuquidar administration was performed one hour before the talinolol administration. Blood, liver and small intestine samples were isolated at 30, 60, 120, 240 and 300 min following talinolol administration ($n = 5$). All blood samples were collected in EDTA tubes. Plasma was isolated from the samples by centrifugation at 4000 rpm for 10 min. Plasma and tissue samples were stored at −80 °C until the day of analysis. After weighing, 1:1–1:3 saline was added to tissue samples and they were homogenized by mechanical homogenizer (Art-Miccra-D1, Germany).

2.4. Analysis of plasma and tissue samples by HPLC/UV

Plasma and tissue concentrations of talinolol were determined by using a validated HPLC/UV method adopted from Oertel et al., 1998, 1994 and Pathak et al., 2010, respectively (Oertel et al., 1998, 1994; Pathak et al., 2010). For HPLC analysis, Waters 2695 HPLC separation module (MA, USA) equipped with a Waters 2487 UV/VIS detector (MA, USA) was used. The chromatographic conditions for the determination of talinolol in plasma and tissues were given in Table 1.

2.5. Pharmacokinetic analysis

Plasma and tissue concentrations of talinolol were calculated from the standard calibration curve. Peak plasma concentration (C_{max}) and time to peak concentration (t_{max}) values were directly obtained from the talinolol plasma/tissue concentration-time curves. The area under the concentration-time curve from 0 to 5 h ($AUC_{0-5\ h}$) was calculated by the trapezoidal method. $AUC_{0-\infty}$ was obtained by addition of the extrapolated part after the last sampling time. Other pharmacokinetic parameters of talinolol were calculated by the non-compartmental method. The elimination rate constant (k_{el}) was calculated from the terminal points of the concentration-time plot and the slope of this line was equal to k_{el}. Terminal elimination half-life ($t_{1/2}$) was calculated by $\ln 2/k_{el}$ equation. The apparent total systemic clearance (CL/F) was calculated by the formula dose/AUC_{total} and the apparent volume of distribution (V_d) by CL/k_{el}.

2.6. Animals for mRNA and protein expression

Mice were sacrificed at ZT3 and ZT15, and liver and small intestine samples were isolated. Left lobe of the liver was used for analysis. Small intestine was washed with cold phosphate buffered saline (PBS) to clear the fecal matter, opened and the mucous layer was scraped. Liver and small intestine samples were frozen in liquid nitrogen and stored at −80 °C until analyzed.

2.7. Gene expression analysis

Liver and small intestine tissues were ground with mortar and pestle

Table 1
Chromatographic conditions for the determination of talinolol in plasma and tissues.

Name	Chromatographic conditions for plasma analysis	Chromatographic conditions for tissue analysis
Column	Phenomenex® Gemini® C_{18} RP column, 5-µm (250 × 4.6 mm)	Phenomenex® Gemini® C_{18} RP column, 5-µm (250 × 4.6 mm)
Precolumn	Phenomenex® Gemini® C_{18} (4 × 3.0 mm)	Phenomenex® Gemini® C_{18} (4 × 3.0 mm)
Mobile phase	KH_2PO_4 buffer: Acetonitrile = 73:27 (v:v) pH = 4	KH_2PO_4 buffer: Acetonitrile = 60:40 (v:v) pH = 3.1.Triethylamine as peak modifier (0.6 ml/L)
Flow rate	1 ml/min	1 ml/min
Column temperature	35 °C	35 °C
Injection volume	45 µl	45 µl
Detector	Waters 2487 UV-detector	Waters 2487 UV-detector
Wavelength	245 nm	245 nm
Retention time	9.9 min	4.7 min
Extraction	Oasis® HLB sample extraction cartridge (1 cc/30 mg)	Tert-butyl-methyl-ether (2 ml)
Internal Standard- (IS) solution (escitalopram)	–	22.5 µg/ml

under liquid nitrogen. RNA isolation was performed using NucleoSpin RNA kit (Macherey Nagel, Germany) according to the manufacturer's recommendations. The amount and purity of RNA obtained were determined in ng/ml by measuring with NanoDrop 8000 (Thermo Fisher Scientific, MA, USA) spectrophotometer device. cDNA synthesis was performed by reverse transcriptase reaction using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, USA). Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed using SensiFast SYBR No-ROX reagents (Bioline Reagent, UK) and the Bio-Rad CFX96 instrument (CA, USA). Primer sequences were as follows: "AAT GTT TCG TTA TGC AGG TTG GC (F)" and "TGG CTC TTT TAT CGG CCT CAC (R)" for *abcb1a*, "GAG ACA GGA TAT AAG CTG GTT CG (F)" and "CGC CCC TTT AAC ACT AGA AGC A (R)" for *abcb1b*. Relative gene expression was calculated and the control gene 36B4 was used for the relative expression calculation.

2.8. Protein expression analysis

Liver tissues were ground with mortar and pestle under liquid nitrogen, then mixed with lysis buffer (RIPA, Santa Cruz, USA). The mixture was sonicated and centrifuged at 14,000 rpm for 30 min at +4 °C. The supernatant was separated into a clean tube. Small intestine samples were mixed with Tris–HCl (0.01 M, pH 7.4) including protease inhibitor cocktail, sonicated and centrifuged at 14,000 rpm for 30 min at +4 °C. The supernatant was discarded and the pellet was mixed with Tris–HCl again. Total protein concentration was measured with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA). 50-µg total protein extract was separated on 10% TGX Stain-Free FastCast gels (Bio-Rad, CA, USA) and transferred to 0.45 µm polyvinyl difluoride membranes (Millipore, MA, USA) at 100 mA for 10 h with cold transfer apparatus (Bio-Rad, CA, USA). The membranes were blocked overnight at +4 °C with Tris-buffered saline plus 0.02% Tween 20 containing 5% nonfat milk. Primary and secondary antibodies were prepared in 1% TBST with 5% nonfat milk as P-gp 1:250, Lamin B1 1:4000, Anti-mouse IgG 1:2000 and Anti-rabbit IgG 1:2000. Primary antibody incubation was performed overnight at +4 °C, and then secondary antibody incubation was performed at room temperature for 1 h. Chemoluminescence detection was performed using LumiGlo ECL kit (CTS, MA, USA) and imaging was achieved with Bio-Rad Chemidoc Gel Imaging System (CA, USA). The grading of the bands was determined with the ImageJ (National Institutes of Health, USA) program.

2.9. Statistical analyses

The statistical significance of differences observed between groups was validated by one-, two- or three-way analysis of variance (ANOVA). All statistical tests were performed using GraphPad Prism version 8.00 for Windows (La Jolla, CA, USA).

3. Results

3.1. Effects of dosing-time and sex on talinolol plasma pharmacokinetics with or without zosuquidar

After intraperitoneal administration of talinolol, plasma C_{max} value was observed at 0.5 h in all groups (Table 2). Dosing-time, sex, and zosuquidar administration did not effect t_{max} or C_{max} in plasma. However, talinolol plasma $AUC_{0–5\ h}$ and AUC_{total} were increased by zosuquidar administration regardless of dosing-time and sex (Three-way ANOVA, $p < 0.05$) (Table 2, Fig. 1). At ZT3, talinolol plasma $AUC_{0–5\ h}$ was increased by 70.52% in females and 38.99% in males by zosuquidar administration. Again at ZT15, zosuquidar administration increased talinolol plasma $AUC_{0–5\ h}$ by 19.5% in females and 26.72% in males. It was seen that the increment was greater at ZT3 especially in

Table 2
Plasma pharmacokinetics of i.p. talinolol with or without zosuquidar administration in male and female mice.

PK Parameters of Tal	ZT3				ZT15				
	Female		Male		Female		Male		
	Tal	Tal + Zsq	Tal	Tal + Zsq	Tal	Tal + Zsq	Tal	Tal + Zsq	p value*
t_{max} (h)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	–
C_{max} (µg/ml)	3.34 ± 0.27	3.96 ± 0.88	3.28 ± 0.58	2.98 ± 0.94	3.26 ± 0.86	4.26 ± 0.68	3.86 ± 0.32	4.64 ± 0.83	ns
$AUC_{0–5\ h}$ (µg.h/ml)	3.63 ± 0.39	6.19 ± 1.26	3.59 ± 0.64	4.99 ± 1.18	4.19 ± 0.85	5.01 ± 0.89	4.79 ± 0.47	6.07 ± 0.72	Treat. $p < 0.05$
AUC_{total} (µg.h/ml)	3.63 ± 0.40	6.28 ± 1.34	3.64 ± 0.69	5.05 ± 1.24	4.21 ± 0.86	5.08 ± 0.94	4.80 ± 0.47	6.10 ± 0.73	Treat. $p < 0.05$
$t_{1/2}$ (h)	0.32 ± 0.01	0.40 ± 0.06	0.43 ± 0.09	0.42 ± 0.08	0.58 ± 0.02	0.68 ± 0.06	0.38 ± 0.01	0.41 ± 0.04	ns
Cl/F (ml/h)	0.09 ± 0.03	0.06 ± 0.01	0.11 ± 0.02	0.08 ± 0.01	0.09 ± 0.04	0.08 ± 0.03	0.1 ± 0.02	0.08 ± 0.03	ns
V_d/F(ml)	0.04 ± 0.02	0.03 ± 0.00	0.07 ± 0.01	0.05 ± 0.01	0.08 ± 0.03	0.07 ± 0.03	0.06 ± 0.01	0.05 ± 0.01	ns

PK: Pharmacokinetics. t_{max}: The time when the maximum concentration is reached. C_{max}: The maximum concentration. $t_{1/2}$: Elimination half-life. $AUC_{0–5h}$: Area under the curve between 0 and 5 h. AUC_{total}: Area under the total curve. k_{el}: Elimination rate constant. Cl/F: Clearance. Vd/F: Distribution volume.

* Three-way ANOVA, functions; ZT, Sex, Treatment (w/o zosuquidar). Treat.: Treatment. Mean ± SEM. ns: not significant.

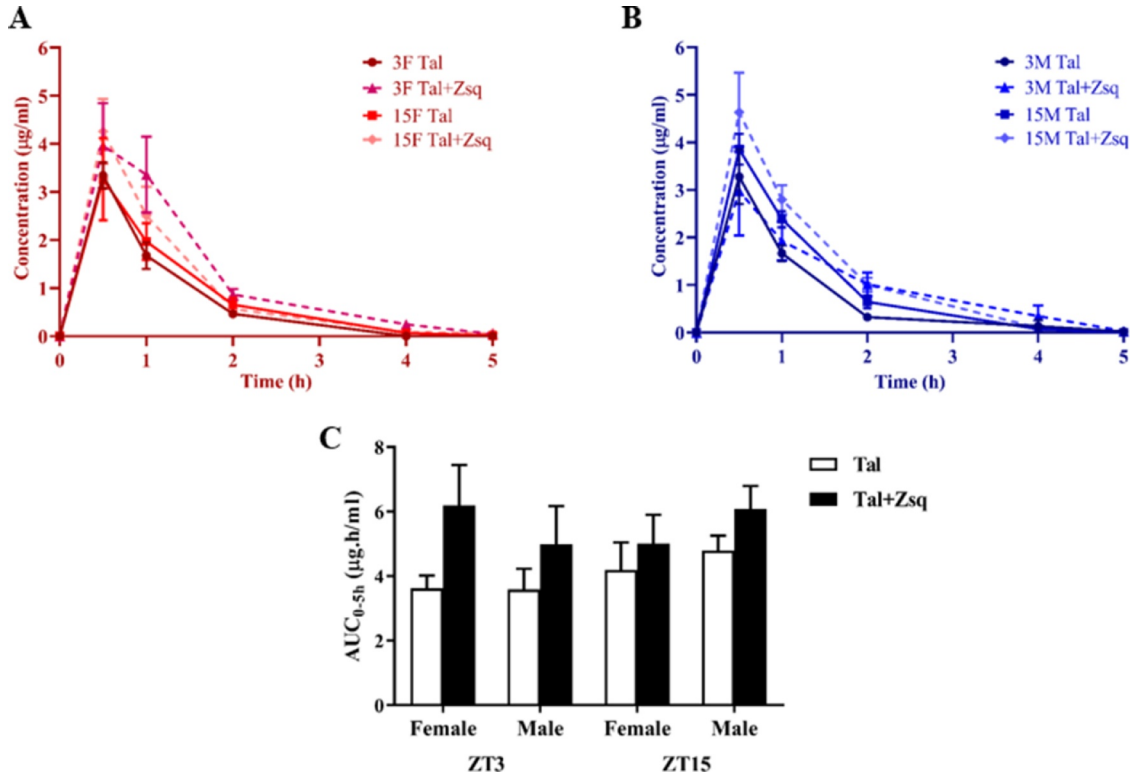

Fig. 1. Plasma concentration-time graphs (A-B) and $AUC_{0-5\ h}$ (C) values of talinolol with or without zosuquidar administration to female (A-C) and male (B-C) mice at ZT3 and ZT15 ($n = 4 - 5$). Tal: Talinolol, Zsq: Zosuquidar, ZT: Zeitgeber Time. Three-way ANOVA. Mean ± SEM.

females.

3.2. Effects of dosing-time and sex on talinolol distribution to liver with or without zosuquidar

Talinolol reached C_{max} at 0.5 h in the liver, as in plasma. Zosuquidar administration increased C_{max} and $AUC_{0-5\ h}$ of talinolol in the liver tissue. The increment of C_{max} and $AUC_{0-5\ h}$ were statistically significant as a function of "Treatment" (Three-way ANOVA, $p<0.05$) (Table 3, Fig. 2). At ZT3, zosuquidar administration increased talinolol liver $AUC_{0-5\ h}$ by 49.66% in females and 41.33% in males. At ZT15, talinolol liver $AUC_{0-5\ h}$ was increased by 23.44% in females and 37.58% in males with zosuquidar administration. As seen in talinolol plasma $AUC_{0-5\ h}$ values, the increament of talinolol liver $AUC_{0-5\ h}$ was greater at ZT3 depending on zosuquidar administration.

3.3. Effects of dosing-time and sex on the intestinal absorption of talinolol with or without zosuquidar

In the intestine, talinolol reached C_{max} at 0.5 h. However, a second increase was seen at 2 h (3 M Tal) and 5 h (3F Tal and 3F Tal + Zsq) in ZT3 groups. $AUC_{0-5\ h}$ of talinolol in the small intestine did not change depending on zosuquidar administration. However, $AUC_{0-5\ h}$ of talinolol was higher at ZT3 in all groups (Three-way ANOVA, $p<0.05$) (Table 4, Fig. 3). In only-talinolol group, $AUC_{0-5\ h}$ of talinolol in the small intestine was higher by 68.16% in females and 123.61% in males at ZT3 compared to ZT15. In zosuquidar administered groups, intestinal $AUC_{0-5\ h\ of}$ talinolol increased by 44.61% in females and 11.11% in males at ZT3 compared to ZT15. The increment was more evident in only-talinolol administered groups.

3.4. Effects of circadian-time and sex on P-gp expression

In the liver and ileum tissues, a time-dependent difference was not observed in mRNA expressions of *abcb1b* in both male and female mice. In the liver, *abcb1a* expression was higher at ZT15 as compared to ZT3 in both females ($p<0.001$) and males ($p<0.01$) (Fig. 4). In the same way, *abcb1a* expression was higher at ZT15 as compared to ZT3 in both female ($p<0.01$) and male ($p<0.0001$) mice in the ileum tissue (Fig. 5). There was no statistically significant difference in P-gp protein expression in female mice in both liver and ileum tissues ($p>0.05$). Liver P-gp protein expression was higher at ZT15 as compared to ZT3 in male mice ($p<0.05$). Significant increases in P-gp protein expression at ZT15 were observed in the ileum tissue in male mice as compared to

Table 3
Liver pharmacokinetics of i.p. talinolol with or without zosuquidar administration in male and female mice.

PK Parameters of Tal	ZT3				ZT15				
	Female		Male		Female		Male		
	Tal	Tal + Zsq	Tal	Tal + Zsq	Tal	Tal + Zsq	Tal	Tal + Zsq	p value*
t_{max} (h)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	–
C_{max} (µg/g)	20.76 ± 6.90	35.03 ± 8.92	17.70 ± 2.76	24.27 ± 7.54	19.54 ± 3.87	25.96 ± 5.46	20.59 ± 1.96	29.38 ± 8.21	Treat. $p<0.05$
$AUC_{0-5\ h}$ (µg.h/g)	28.09 ± 7.65	42.04 ± 7.07	22.45 ± 4.97	31.73 ± 10.92	28.75 ± 4.24	35.49 ± 6.91	28.39 ± 4.05	39.06 ± 6.94	Treat. $p<0.05$

PK: Pharmacokinetics. t_{max}: The time when the maximum concentration is reached. C_{max}: The maximum concentration. $t_{1/2}$: Elimination half-life. AUC_{0-5h}: Area under the curve between 0 and 5 h.

* Three-way ANOVA, functions; ZT, Sex, Treatment (w/o zosuquidar). Treat.: Treatment. Mean ± SEM. ns: not significant.

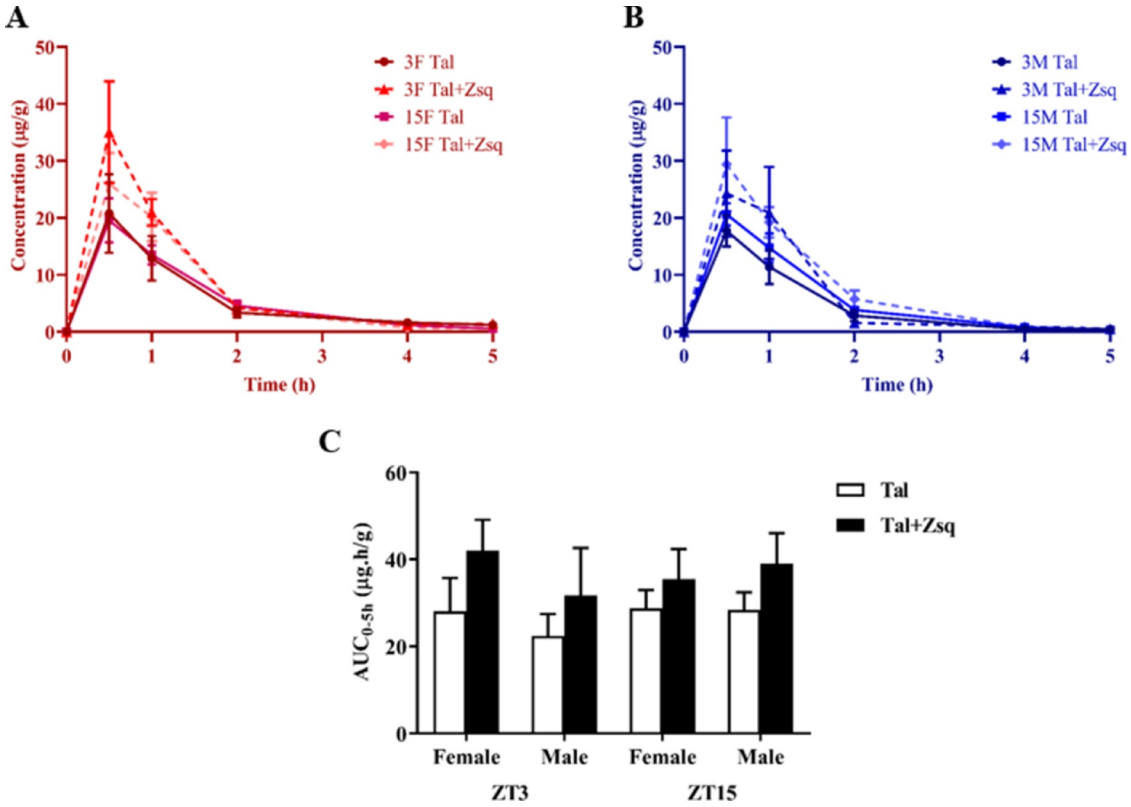

Fig. 2. Liver concentration-time graphs (A-B) and $AUC_{0-5\ h}$ (C) values of talinolol with or without zosuquidar administration to female (A-C) and male (B-C) mice at ZT3 and ZT15 (n = 4–5). Tal: Talinolol, Zsq: Zosuquidar, ZT: Zeitgeber Time. Three-way ANOVA. Mean ± SEM.

Table 4
Ileum pharmacokinetics of i.p. talinolol with or without zosuquidar administration in male and female mice.

PK Parameters of Tal	ZT3				ZT15				
	Female		Male		Female		Male		
	Tal	Tal + Zsq	Tal	Tal + Zsq	Tal	Tal + Zsq	Tal	Tal + Zsq	p value*
t_{max} (h)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	–
C_{max} (µg/g)	12.09 ± 3.44	12.87 ± 0.96	12.33 ± 2.63	10.15 ± 0.51	10.35 ± 2.30	12.39 ± 2.49	10.78 ± 0.74	9.39 ± 2.33	ns
$AUC_{0-5\ h}$ (µg.h/g)	34.81 ± 7.59	31.67 ± 4.75	46.22 ± 12.32	26.49 ± 3.39	20.70 ± 3.21	21.90 ± 4.58	20.67 ± 3.96	23.84 ± 5.65	ZT: $p < 0.05$

PK: Pharmacokinetics. t_{max}: The time when the maximum concentration is reached. C_{max}: The maximum concentration. $t_{1/2}$: Elimination half-life. AUC_{0-5h}: Area under the curve between 0 and 5 h.

* Three-way ANOVA, functions; ZT, Sex, Treatment (w/o zosuquidar). Treat.: Treatment. Mean ± SEM. ns: not significant.

ZT3 ($p < 0.05$) (Fig. 4, Fig. 5).

4. Discussion

Since P-gp function is critical for drug detoxification, circadian-dependent and sex-specific alterations in its expression and activity may account for the inter-individual and dosing time-dependent differences in drug pharmacokinetics thereby in the efficacy and/or toxicity of drugs. Modulation of drug administration time according to the circadian rhythms in P-gp considering the intra- and interindividual variability may be a useful strategy to improve the pharmacological profile of drugs, especially anticancer agents which are P-gp substrates. The intestinal expression of P-gp is affected by the circadian organization of molecular clockwork, providing a link between the CTS and xenobiotic detoxification (Murakami et al., 2008). In recent studies, P-gp mRNA expression and protein levels have been shown to display 24-hour variation in liver and intestinal mucosa in rodents (Ando et al., 2005; Okyar et al., 2019, 2012; Stearns et al., 2008; Zhang et al., 2009). Ando et al., 2005, reported that mRNA expression of *abcb1a* showed clear 24-hour rhythmicity in the liver and intestine of male C57BL/6 J mice with a peak in the latter half of the light phase (ZT12) and a trough at the onset of the light phase (ZT0). In accordance with Ando et al., 2005, we showed that *abcb1a* gene expressions were higher in both liver and ileum tissue, in male and female mice in the activity phase (ZT15) compared to the resting phase (ZT3). In liver and ileum tissues, there were no time-dependent differences in *abcb1b* mRNA expressions in both female and male mice (Ando et al., 2005).

Protein levels of P-gp have also been shown to be rhythmic in the intestines. Protein levels increased towards the end of the light phase and reached their maximum at the beginning of the dark phase. It was detected at the lowest level at the beginning of the light phase (Ando et al., 2005; Okyar et al., 2019; Murakami et al., 2008; Zhang et al., 2009). In our study, similar rhythms at the level of P-gp protein in both female and male mice in ileum tissue were revealed as P-gp protein levels in ZT15 were higher than ZT3 in ileum tissue. While Ando et al., 2005 showed that there was no time-dependent change in P-gp protein levels in the liver, we observed a significant increase in P-gp protein levels only in male mice at the dark (activity) phase in our study.

P-gp expression has also indicated sex-specific changes. Studies have shown that gonadal steroids regulate P-gp expression and function (Fedoruk et al., 2004; Kim and Benet, 2004; Mutoh et al., 2006). In a

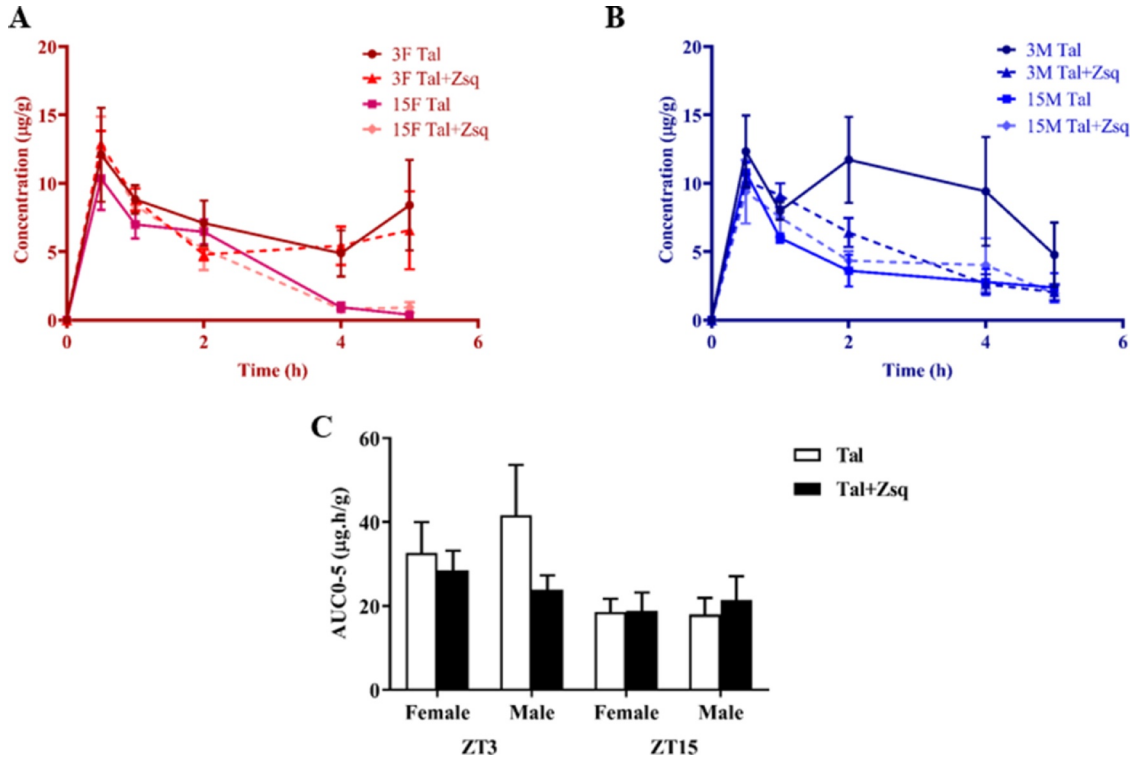

Fig. 3. Ileum concentration-time graphs (A-B) and $AUC_{0-5\ h}$ (C) values of talinolol with or without zosuquidar administration to female (A-C) and male (B-C) mice at ZT3 and ZT15 (n = 4–5). Tal: Talinolol, Zsq: Zosuquidar, ZT: Zeitgeber Time. Three-way ANOVA. Mean ± SEM.

recent study, sex-specific and circadian time-dependent changes were observed in P-gp ileum mRNA expression and protein levels, in which circadian amplitudes were larger in females as compared to males. In liver, P-gp expression was statistically different in female (Okyar et al., 2019). In our study, in contrast, the levels of P-gp protein in the ileum tissue was higher in male mice compared to females in the activity (dark) phase, whereas in the liver, there was an increase in P-gp protein levels only in male mice.

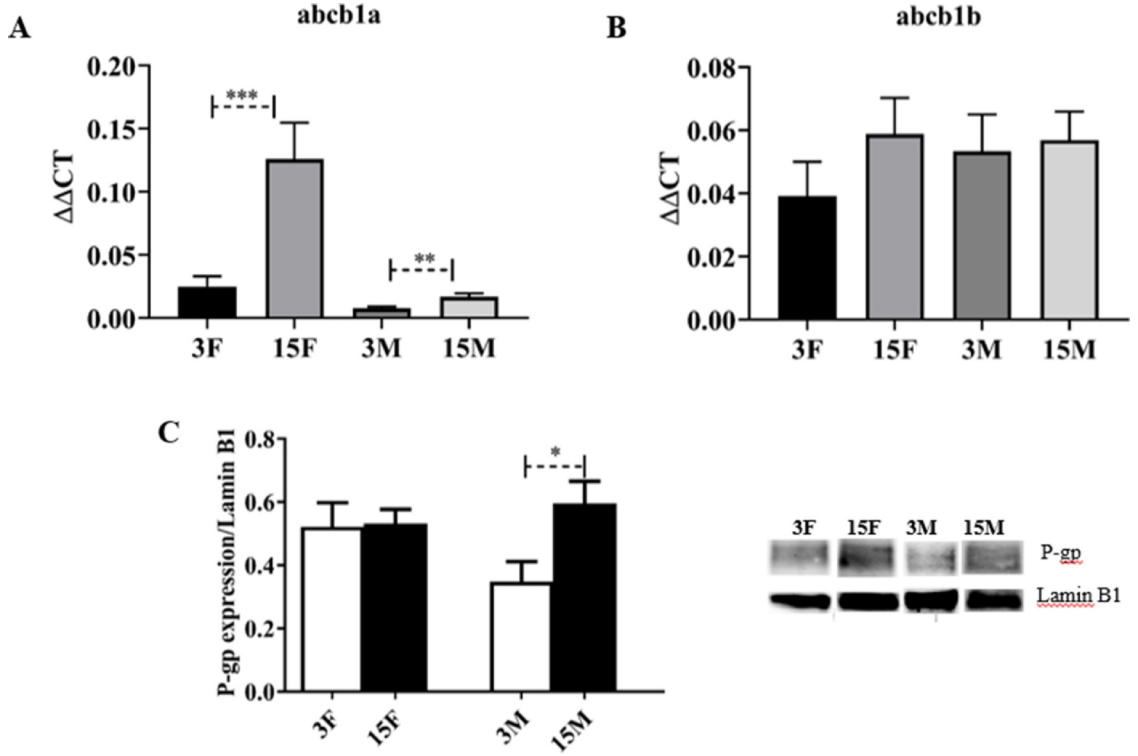

Fig. 4. Liver *abcb1a* (A) ve *abcb1b* (B) mRNA and P-gp protein (C) levels in female and male control mice at ZT1 and ZT13 (n = 4–5). Two-way ANOVA. Mean ± SEM.

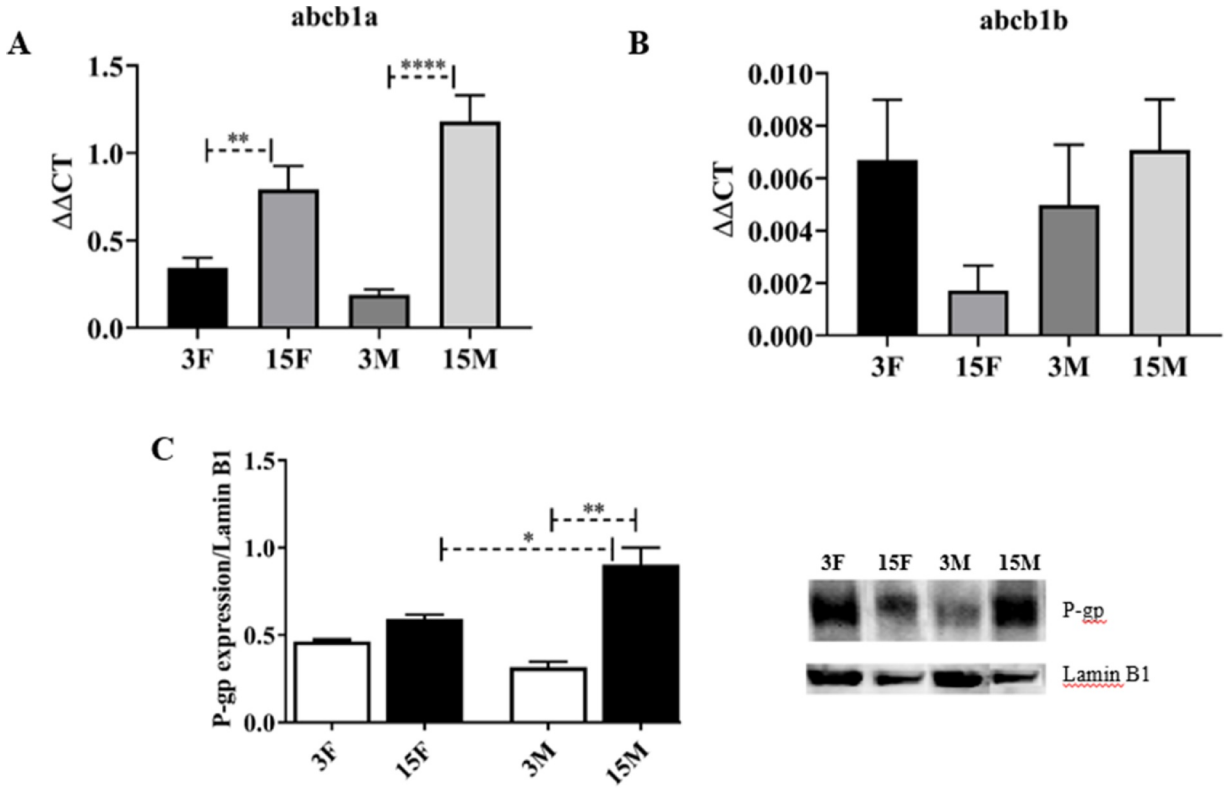

Fig. 5. Ileum *abcb1a* (A) ve *abcb1b* (B) mRNA and P-gp protein (C) levels in female and male control mice at ZT1 and ZT13 (n = 4–5, pooled). Two-way ANOVA. Mean ± SEM.

To date, time-dependent role of P-gp in pharmacokinetics especially in the absorption of drugs has been demonstrated by many studies (Bruguerolle et al., 2008; Lévi et al., 2010; Levi and Schibler, 2007; Ohdo, 2010, 2007). However, with a parenteral administration rather than oral, how P-gp modulation changes the pharmacokinetics of its substrates depending on dosing time has not been fully elucidated. In this study, we aimed to reveal how the function of P-gp changes with time and sex depending as a result of i.p administration of specific P-gp substrate talinolol and a specific inhibitor zosuquidar to mice.

In this study, following i.p. administration, C_{max} level of talinolol was reached at 0.5 hour (t_{max}) in plasma, liver and ileum in each group. Zosuquidar administration did not change plasma and tissues t_{max} of talinolol in any group. Talinolol plasma $AUC_{0-5\ h}$ and AUC_{total} were increased with zosuquidar administration regardless of dosing-time and sex (Three-way ANOVA, $p < 0.05$). While zosuquidar administration did not change plasma C_{max} levels significantly in all groups, the increase in AUC levels can be explained by a decrease in the elimination of talinolol from plasma. Although not statistically significant, zosuquidar reduced clearance in both male and female mice at all ZT. It was reported that about 60% of talinolol and its metabolites were excreted by kidney and about 40% extrarenally, mainly in faeces (Trausch et al., 1995). However biliary secretion of the intravenous dose of talinolol is less than 10% (Oswald et al., 2011; Terhaag et al., 1989; Weitschies et al., 2005). The total AUC values of the talinolol administered parenterally did not change significantly depending on dosing time. The reason can be explained by the fact that the expressions of P-gp do not show a circadian rhythm in the kidneys, which is the main elimination route (Ando et al., 2005).

Zosuquidar increased talinolol plasma $AUC_{0-5\ h}$ by 70.52% in females and 38.99% in males at ZT3, however 19.5% in females and 26.72% in males at ZT15. Although P-gp protein level was higher at ZT15 (activity phase) than ZT3 (resting phase), it was seen that the inhibitor's effect was greater at ZT3 especially in females. It is expected that inhibition of P-gp should be stronger at activity phase when the P-gp protein expression is higher. As parallel with our results, Okyar et al., 2012 showed that P-gp inhibitor vinblastine changed the effective permeability and was doubled in both jejunum and ileum as compared to control groups *in situ* intestinal perfusion study in rats. Ileum perfusions displayed 83.7% enhancement during the daytime and 124.7% increment during the nighttime by vinblastine. In this study, where P-gp expression was increased, the inhibitory effect was more evident *in situ* conditions, while in systemic administration, the effect of the inhibitor was not so pronounced in the period when the P-gp expression phase was higher (Okyar et al., 2012). It has been already known that basal bile flow is higher during the night than day span in rats (Ho and Drummond, 1975) and glomerular filtration rate (GFR) and renal blood flow are rhythmic, being higher at night (activity span) in rodents (Pons et al., 1994; Rebuelto et al., 2004). Hence, considering all these pathways, the zosuquidar may exhibit a higher clearance at night.

When talinolol administered by i.p., $AUC_{0-5\ h}$ of talinolol in the plasma was higher by 33% in males and slightly higher in females at ZT15 compared to ZT3. It was shown that the plasma exposure of talinolol was reduced in ZT15 by orally administered talinolol (Okyar et al., 2019). In this context, it has been demonstrated that the pharmacokinetic profile depending on time varies by application route. Circadian changes in P-gp levels in the ileum tissue affect the absorption of orally administered substrates. However, no dramatic change was observed duo to direct intestinal secretion with parenteral administration of substrate drug talinolol. Talinolol is a substrate and its bioavailability is limited especially by P-gp due to intestinal secretion. However, only 2% of talinolol administered intravenously has been shown to be eliminated in the small intestine segment (Spahn-Langguth et al., 1998). Matsuda et al. (2013) showed that oral administration of zosuquidar was considered to inhibit both systemic and intestinal P-gp, while i.v. administration of zosuquidar inhibited only systemic P-gp expression in liver, kidney, and other organs, except for intestine. Fexofenadine bioavailability after oral administration of

zosuquidar was 4-fold higher than control but was almost the same as control after i.v. administration despite a significant change in systemic clearance. This finding can also explain our data, since $AUC_{0-5\ h}$ of talinolol in the small intestine did not change depending on zosuquidar administration.

The talinolol levels in the liver had a similar pattern to that in the plasma, and the increased uptake of talinolol by tissue can be explained by the decreased plasma clearance of talinolol in the presence of zosuquidar. As seen in talinolol plasma $AUC_{0-5\ h}$ values, the increment of talinolol liver $AUC_{0-5\ h}$ was greater at ZT3 depending on zosuquidar administration. Although there was no significant increase in ZT15 plasma levels, $AUC_{0-5\ h}$ of talinolol was higher at ZT3 in all groups in ileum tissue (Three-way ANOVA, $p < 0.05$). Only talinolol administered groups compared by ZT, $AUC_{0-5\ h}$ of talinolol in the small intestine was higher by 68.2% in females and 123.6% in males at ZT3 compared to ZT15. The difference between ZT3 and ZT15 in P-gp protein expression in ileum tissue, especially in male mice, was more pronounced than in female mice. P-gp protein expression in male mice at ZT3 was significantly lower than ZT15. The low exposure of talinolol at ZT15, where P-gp protein expression was higher, can be explained by the fact that the talinolol was effluxed by P-gp from the small intestinal tissue to the lumen. Ando et al., 2005, showed that whether the daily variation of *abcb1a* expression could cause a change in the intestinal accumulation of digoxin, a P-gp substrate, ex vivo during 24 h. Similar with our results, the mean digoxin concentration at ZT12 was significantly ($p < 0.05$) lower than it was at ZT0.

Talinolol has a double-peak phenomenon in plasma after oral administration in rodents (Ozturk et al., 2017; Weitschies et al., 2005). Recently Okyar et al., 2019, showed a second peak in talinolol plasma concentration-time profiles, which was observed 2 h after oral administration of drug in fasted male mice treated at ZT3. However, we observed a second increase in ileum talinolol concentration at 2 h and 5 h in ZT3 groups but not in plasma concentration-time profile after i.p. administration. Talinolol has a side-dependent absorption in rodents. MacLean et al., 2008 showed that P-gp protein levels increased along the intestine from duodenum to ileum. In this context, intestinal secretion of talinolol by P-gp may contribute to the double-peak phenomenon and may be the cause of the decreased oral bioavailability of this drug, as well. In our study first C_{max} was seen at 0.5 h in ileum. After intestinal secretion, talinolol may be reabsorbed from intestinal lumen when P-gp activity was low.

In our study, there is 1 hour difference between zosuquidar and talinolol administration. Kemper et al. (2004) showed that after i.v. administration, the plasma concentration-time curve followed biexponential decay kinetics with distribution and elimination half-lives of 15 min and 2.1 h, respectively. When zosuquidar was administered i.v. 10 min before paclitaxel, the paclitaxel levels in the brain of wild-type mice increased by 5.6-fold, whereas the increase was only 2.1-fold when zosuquidar was administered 1 h before paclitaxel. This suggests that the inhibition of P-gp at the blood-brain barrier by zosuquidar is rapidly reversible, and the concentrations of zosuquidar in the plasma have already declined to the levels insufficient to inhibit P-gp at the blood-brain barrier. But this difference has not been so dramatically reflected in plasma AUC values. Although it was administered 1 hour ago, inhibitory effect of P-gp continued (Kemper et al., 2004). Long-lasting inhibitory effect of P-gp has also been reported in an in vitro study where zosuquidar inhibited P-gp in multidrug-resistant tumor cells, even after it was removed from the culture medium for several hours (Dantzig et al., 1999).

In our study, P-gp protein levels in liver and ileum tissues were not different in female mice but higher in ZT15 as compared to ZT3 in male mice. There was no statistically significant difference in talinolol concentration depending on time and sex in the plasma and liver however there was significant time dependent difference in ileum $AUC_{0-5\ h}$ of talinolol. Talinolol plasma and liver $AUC_{0-5\ h}$ were increased by zosuquidar administration regardless of dosing-time and sex.

This study showed that P-gp modulation may vary depending on route and time of drug administration. It is important to determine the transporter dependent drug-drug interaction studies and their outcomes according to choronomodulated applications. Our study findings are considerable in terms of revealing changes in pharmacokinetic profiles of P-gp substrates due to the time of administration in combination with P-gp inhibitors/modulators in managing polypharmacy. Since, variations in pharmacokinetics may also cause changes in toxicity and pharmacodynamic profiles of drugs. Translation of these findings from preclinical to humans can give promises towards personalized medicine with patient's sex, polymorphisms and chronomodulated application of drugs.

CRediT authorship contribution statement

Zeliha Pala Kara: Conceptualization, Investigation, Funding acquisition, Writing - original draft. **Dilek Ozturk Civelek:** Investigation, Formal analysis, Writing - original draft. **Narin Ozturk:** Investigation, Writing - original draft. **Alper Okyar:** Conceptualization, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this study.

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