The role of Pgp in zebrafish cardiac glycosides chronotoxicity

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Abstract

Introduction: Circadian rhythms influence susceptibility and responses of organisms to xenobiotic exposure. It is well known that ATP binding cassette (ABC) transporters have a major influence on bio-availability, metabolism and excretion of drugs. Recently it has been shown that the gene expression of some transporters, including P-glycoprotein (Pgp), is under circadian transcriptional regulation. It has been hypothesized that Pgp expression might contribute to the observed diurnal variation in cardiac glycosides toxicity in human. Accordingly, the aims of this study were to establish a cardiac glycosides chronotoxicity model using zebrafish and to investigate the role of Pgp expression in observed diurnal changes in toxicity by using pharmacological Pgp inhibitors.

Materials and Methods: Three or eight days post fertilization (dpf) zebrafish were incubated for 3 hours with digitoxin at two different time points: at the beginning (Zeitgeber, ZT 1-4) and at the end (ZT 10-13) of the light cycle. In the case of Pgp inhibitory studies, 3dpf or 8 dpf fish at two different circadian time points were pre-soaked for 1 hour with verapamil (only 3dpf embryos) or ketoconazole before 3h incubation with digitoxin. Toxicity was assessed using newly developed toxicity scoring systems based on cardiovascular function and phenotypic characteristic. Furthermore, qRT-PCR was performed to measure gene expression levels of the gene for Pgp MDR1.

Results: Diurnal variation in digitoxin toxicity was demonstrated on 3 and 8 dpf zebrafish: significantly higher toxicity occurred at ZT 10-13 versus ZT 1-4. Additionally, a trend toward reduced expression levels of MDR1 in 3 and 8dpf fish ZT 13 in comparison with ZT 4 was observed. Statistically significant increase in toxicity was observed when zebrafish were pre-soaked with ketoconazole before incubation with digitoxin at ZT 1-4. Moreover ketoconazole was found to be toxic for 8 dpf fish at ZT 10-13, something that was not observed at ZT 1-4. Verapamil in the tested concentration did not cause an increase in digitoxin toxicity.

Discussion: The present study highlights the suitability of zebrafish as a model to perform cardiac glycosides chronotoxicity studies, since a significant diurnal difference in digitoxin toxicity has been demonstrated on 3 and 8 dpf zebrafish. The observed augmented toxicity of digitoxin with ketoconazole could be explained by inhibition of both Pgp and CYP3A, which cannot be differentiated in the present study. Although the diurnal difference in MDR1 expression between ZT 4 and ZT 13 was not found to be significant, the general fluctuation trend in MDR1 expression levels corresponded well with variation in toxicity manifestation. Also, gene expression levels not always represent actual activity of the transporter. More studies need to be performed to unveil the actual contribution of Pgp and cytochrome P450 in observed diurnal changes in cardiac glycosides toxicity.

Keywords: zebrafish, P-glycoprotein, cardiac glycosides chronotoxicity

List of abbreviations

ABC	ATP binding cassette
BBB	Blood-brain barrier
DMSO	Dimethyl sulfoxide
Dpf	Days post fertilization
DT	Digitoxin
KT	Ketoconazole
LD	Light/dark
Pgp	P-glycoprotein
SEM	Standard error of the mean
VP	Verapamil
ZT	Zeitgeber

Outline

I. Introduction

The influence of the circadian rhythm on susceptibility and responses of organisms to external agents, such as food, toxins and drugs, have been extensively studied during the last decades and have become an important field of consideration for toxicity studies and assessments (1-2). Chronopharmacology studies the influence of the circadian rhythm on kinetics and dynamics of pharmaceuticals (3). Accordingly, absorption, distribution and elimination of certain pharmaceuticals can vary during the day, which consequently can affect their efficacy and toxicity (3-4).

It is well known that ATP binding cassette (ABC) transporters have a major influence on bio-availability, metabolism and excretion of drugs. The penetration of certain drugs across biological membranes greatly depends on ABC transporter expression. These transporters are important detoxification and protection mechanisms. By pumping drugs out of the cells, ABC transporters determine to a large extent the clinical effectiveness, side effects and toxicity of drugs (5, 6). P-glycoprotein (Pgp) is well known as a multi-drug resistance protein, encoded in humans by genes MDR1 and MDR3. It is abundant in the liver, intestine and kidney, contributing to drug absorption and elimination (7). An important role of Pgp in limiting xenobiotics absorption has already been shown in many studies. Recently it has been shown in mouse models that gene expression of some transporters, such as Pgp, MRP2, OCT1, is under circadian transcriptional regulation (7,8).

In a study performed by *Ando et al, 2005,* mice experiments showed that MDR1 transcript level, Pgp expression, and Pgp function exhibit a daily rhythm. Obtained results demonstrated a significantly higher Pgp expression at the end of a light cycle versus the starting point of a light cycle (7).

Furthermore, a recently conducted prospective study in Sri Lanka on a large cohort of people poisoned with Thevetia peruviana (yellow oleander) seeds showed diurnal variation in toxicity manifestation (9). The yellow oleander is a common tropic tree that contains cardiac glycosides, i.e. Thevetin A, Thevetin B etc. Oleander glycoside toxicity on cardiac muscle is similar to that of digitalis glycosides used in clinical practice: digoxin and digitoxin, due to a common mechanism of action by inhibiting Na^+/K^+ ATPase pump (10, 11). Digitalis cardiac glycosides have been found to be a substrate for Pgp in several *in vitro* human and animal studies as well as in vivo animal

studies. Accordingly, it has been shown that Pgp plays an important role in limiting digoxin absorption (12, 13, 43). Therefore, it was hypothesized that increased intestinal Pgp activity in the evening is responsible for the toxicity mitigation observed in Sri Lanka study possibly due to a reduction in oleander glycosides absorption.

Therefore, a better understanding of periodic and predictable changes in drug efficacy and toxicity will solve problems of drug optimization regarding effectiveness and tolerance, timing and dosing.

Currently, there are several in vitro and in vivo models that are used for toxicity studies (14). Yet, *in vivo* mammalian models require the use of large numbers of animals. In turn, the use of invertebrate models or *in vitro* methods, such as cell cultures, results in toxicity being studied in a non-physiological setting (15-16). Therefore, other *in vivo*, *in vitro or ex vivo* models should be used for toxicity studies.

Zebrafish (*Danio rerio*) models, which were used in our study, have already been proved to be efficient and functional in various pharmacological and toxicological studies (15,17-18). It is a vertebrate model organism that due to a short reproductive cycle, production of large number of transparent embryos, ability to absorb compounds through water and low cost became an attractive alternative model for toxicity testing of pharmaceuticals and functioning of physiological systems (14-15, 17). Furthermore, zebrafish organs, such as heart, brain, liver, intestine, kidney, are similar to human both by morphology and function (18).

Remarkably, all the basic architecture of a zebrafish' embryonic heart, as well as a matured blood circulatory system, can be observed already at 48 hpf (15,19-20). This advantage, together with the fact that embryonic zebrafish heart development resembles that of humans, makes zebrafish an excellent model to study human cardiovascular diseases. Owing to the high similarity in cardiovascular morphology and function, as well as availability of zebrafish orthologs of human genes with high conservation of gene function compared with humans, the zebrafish presents an excellent model for cardiovascular toxicology studies (18-19, 21). Additionally, it was shown that numerous phenotypes of zebrafish mutations resemble human diseases (17-18).

Moreover, Pgp-encoding homologous genes as well as the protein itself were found to be expressed in many aquatic animals, including *Danio rerio*. Furthermore, the Pgp

tissue distribution in zebrafish exhibits a similar pattern to that of mammals: high expression of Pgp in the liver, intestine, BBB, kidney and pancreas. Additionally, it has been shown that PXR transcriptional regulation of CYP3A and MDR1 is conserved in zebrafish (16).

The lack of studies in chronobiology, as well as chronopharmacology and chronotoxicology drove us to set up an appropriate *in vivo* model in order to provide novel insights into mechanisms of these phenomena.

With an established zebrafish chronotoxicity model it would become possible to unveil underlying reasons for various chronopharmacological phenomena and as a result to test different compounds and their possible antidotes in *in vivo* physiological systems. Zebrafish embryos are permeable to many small molecules: compounds are absorbed through the skin and gills, which makes toxicity studies efficient and easy to perform (17-18).

In our study we created a cardiac glycosides toxicity model by testing digitoxin, one of the most frequently used drugs in clinical practice along with its metabolite digoxin, on 3 and 8 dpf zebrafish . The toxicity was evaluated by using a toxicity scoring system based on cardiovascular function and phenotypic characteristic. Furthermore, in this study we tried to influence the toxicity manifestation by inhibiting Pgp with verapamil (VP) and ketoconazole (KT).

The aims of this study were to establish a cardiac glycosides chronotoxicity model using zebrafish and to investigate the role of Pgp expression in observed diurnal changes in toxicity by using pharmacological Pgp inhibitors.

II. Materials and Methods

General

2.1 Chemicals

Digitoxin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Verapamil was procured from Acros organics (New Jersey, USA). Ketoconazole was purchased from TOCRIS bioscience (Ellisville, MO, USA). Stock solutions of each compound were made in the solvent dimethyl sulfoxide, DMSO (Sigma-Aldrich, St. Louis, MO, USA).

2.2 Animals

Wild type line (WIK) zebrafish *(Danio rerio)* were housed in the Queen's Medical Research Institute (BRR) zebrafish facility of the University of Edinburgh in a temperature- and humidity-controlled room with a 14:10-h light/dark (LD) cycle in 10 L tanks with flow-through freshwater at aquarium-maintained conditions: pH ~7.8, temperature ~29ºC conductivity ~384µS and system and system dissolved oxygen ~7.5 mg/l, as described previously (22-23). Adult fish were adapted to a 14:10-h LD cycle: with lights on at 9:00, and lights off at 23:00. For convenience, several tanks were moved to a cabinet with a different 14:10-h LD cycle: with lights on at 00:00, and lights off at 14:00. The fish were allowed to acclimatize for at least four weeks to the new LD cycle before experiments started.

All experiments carried out in this study were conducted humanely and according to the Animals Act (Scientific procedures) 1986. The research project was approved by Home Office, UK.

2.3 Embryo collection and maintenance

Zebrafish embryos were collected from natural random mating of fish with two different LD cycles respectively and maintained at room temperature (~28.5ºC) in system water with antiseptic methylene blue (methylthioninium chloride, 0.5mg/l) under the standard conditions and a corresponding LD cycle. After 5 dpf, fish were moved to bigger tanks with ~ 0.3 L system water and housed under standard conditions and a feeding regime.

By three days after fertilisation zebrafish embryos have all major organs and functioning circulatory system (15,19-20,24). As mentioned before, it has been hypothesized that Pgp might be involved in the observed chronotoxicological

phenomenon in oleander poisoning case (9). However, in previously published studies it has been shown that Pgp is not expressed in zebrafish brain before 5dpf and becomes fully functioning only by 7 dpf (25-26). Therefore, zebrafish embryos were tested at two different developmental stages: 3 and 8 dpf. Accordingly, 3 or 8 dpf embryos were used in present experimental studies. Embryos were cleaned daily by changing the system water and removing any dead material and debris.

2.4 Incubation of zebrafish embryos

In pilot dose-response studies 3 dpf zebrafish embryos were incubated with digitoxin for 3 h in 24-well plates (Greiner Bio-One, Germany) at 28,5°C. Accordingly, 7 embryos were carefully transferred with minimum amount of water to each well containing 1 ml methylene blue system water with the solvent DMSO (concentration during incubation 1%) or digitoxin [1.375, 3.75, 7.5, 10, 12.5, 15, 17.5, 30, 60 µM]. Additionally, dose-response studies for verapamil and ketoconazole were performed.

Chronotoxicity studies were performed using zebrafish embryos at two developmental stages: 3 and 8 dpf. Accordingly, 3 or 8 dpf zebrafish embryos were incubated for 3 h in 24-well plates at 28,5°C at two different time points: at the beginning and the end of their light cycle Zeitgeber (ZT) 1-4 and ZT 10-13: where ZT 0 is defined as lights on and ZT 14 as lights off. Accordingly, 6-7 embryos were carefully transferred to each well containing 1 ml methylene blue system water with the solvent DMSO (concentration during incubation 1%) or digitoxin [for 3 dpf fish: 3.75, 7.5, 10, 12.5, 15, 17.5, 30 µM and 8dpf fish: 7.5 µM] as described above. The decision regarding which doses should be used in chronotoxicity studies was made based on results obtained from 3h-incubation dose-response studies on 3 dpf embryos.

Verapamil has been already used as a Pgp inhibitor in zebrafish studies (25,26). Ketoconazole is well known for its potential drug-drug interactions with different medicines which are Pgp and CYP3A4 substrates, including cardiac glycosides, which increases their absorption and prolongs elimination (38). Moreover, Pgp's inhibitory activity of ketoconazole has been shown in several *in vitro* as well as *in vivo* studies (39-40). Unfortunately, no studies on zebrafish were previously conducted with ketoconazole to test its inhibitory activity on Pgp and find a proper dose. However, in *in vitro* studies on MDR1-MDCK type II cells expressing human Pgp, it was demonstrated that ketoconazole is a more potent inhibitor when compared to verapamil; belonging to the same type of Pgp inhibitors (nontransported substrates), ketoconazole has lower IC50 (39).

Accordingly, in case of Pgp inhibitory studies, 3 dpf embryos with two different circadian rhythms were pre-soaked for 1 hour with verapamil [50μM] or ketoconazole [100μM]. Directly after, embryos were rinsed in fresh system water to wash out medium with verapamil or ketoconazole and 7 embryos were transferred to each well containing 1 ml methylene blue system water with digitoxin [for VP studies: 7.5 and 15 μM, for KT studies: 7.5 μM] as mentioned above and incubated for 3 hours. At the same time 7 untreated embryos were transferred in the same manner to each well containing 1 ml methylene blue system water with digitoxin [for VP studies: 7.5 and 15 μM, for KT studies: 7.5 μM] and incubated for 3 hours. Also, DMSO and verapamil [50μM] or ketoconazole [100uM] were included as control groups in all studies accordingly. The decision regarding digitoxin doses that should be tested in chronotoxicity studies with pharmacological Pgp inhibitors was made based on data derived from chronotoxicity studies described above.

Similar studies with the Pgp inhibitor ketoconazole were performed on 8 dpf embryos, using the same concentration of digitoxin [7.5 μM] and lower concentration of ketoconazole [70 μM] due the considerable toxicity of higher doses for 8dpf fish both at ZT 1-4 and ZT 10-13. Due to a lack of time and zebrafish embryos, it was not possible to test different concentrations of digitoxin on 8dpf fish, as well as to test different Pgp inhibitors.

Sample analysis

2.5 Toxicity assessment

The entire zebrafish embryo heart is well within the range of microscopic examination due to embryo's transparency (15). According to the Home office requirements, death cannot be used as an outcome in adult zebrafish toxicity studies. Therefore, a new toxicity scoring system was developed and compared with the toxicity assessment based on mortality. Respectively, the toxicity level in 3 dpf embryos studies was estimated by two different approaches: lethality rate (absence of heart beat/ a faint shudder of atrial and ventricular walls and absence of blood circulation), and using a zebrafish toxicity scoring system as shown below (Table 1): assessing alterations in heart beat and blood circulation (tail blood flow velocity). These criteria where chosen based on published approaches for cardiotoxicity assessment in zebrafish (15,17).

Table 1. Toxicity scoring system for 3 dpf zebrafish embryos

N	Criteria	Score 0	Score 1	Score 2
1	Heart rate	Normal (~150 b/m)	Significantly slower than normal (~ two times slower)	None
2	Tail blood circulation	Normal	Slow (~ two times slower)	None
* Maximum score 4, minimum score 0				

Moreover, several cases of pericardial oedema which is another indicator of cardiac toxicity and ventricular stand still were recorded (15,17). However, due to the short time of incubation and rarity of these pathological phenomena, we decided not to include these criteria into our scoring system.

Due to the lack of transparency of 8dpf embryos different criteria were used to assess toxicity in 8dpf zebrafish studies, as shown below (Table 2). Fish were scored hourly during incubation time.

Table 2. Toxicity scoring system for 8 dpf zebrafish embryos

N	Criteria	Score 0	Score 1	Score 2
1	Active movement	Active	-	-
2	Response to stimuli	-	Poor	None
3	Tail blood flow	Normal	Slow (<~ 2times slower than normal)	None
4	Body position	Normal	-	Abnormal
*Maximum score:6, minimum score 0				

2.6 RNA-isolation

In several previous studies on zebrafish, it has been shown that for RNA extraction a pool from 10-25 embryos per sample has to be used to get sufficient amount of RNA (27-28). We found that 15 pooled 3 dpf embryos, 12 pooled 8 dpf and 10 pooled 10pdf embryos gave enough RNA to perform reverse transcription and qRT-PCR afterwards. Therefore, appropriate amount of embryos from each experimental group were pooled together and placed in 400 µL RNAlater RNA stabilization reagent (QIAGEN, Hilden, Germany). Samples were incubated overnight at 2-8°C and afterwards transferred to -

80°C for later analyses. Samples from at least three chronotoxicity studies carried out on different days were used for qRT-PCR analysis.

When ready to analyze, samples were mechanically homogenized in 600µL buffer RLT using a homogenizer (Retsch, Germany) for 45 sec at 30Hz. RNA was isolated using the RNeasy Mini kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany) including a DNase 1 treatment step (QIAGEN, Hilden, Germany). RNA concentrations were quantified by using NanoDrop (NanoDrop Products, USA).

2.7 Primer design

In human, Pgp is known to be encoded by MDR1/ABCB1 subfamily and MDR3 (7). Yet, it has been reported that ABCB5 is homologous to other ABC transporter genes, i.e. ABCB1 (30). In addition, it has been reported that aminoacid sequence of human Pgp ABCB5 is on 73% homologous to the known Pgp isoforms ABCB1 (MDR1) (29). Therefore, to study the expression of MDR1 in zebrafish, the full ABCB transporter MDR1/ABCB5 was chosen, since only for this MDR1 subfamily the gene sequence is known for zebrafish. Moreover, MDR1 is tentatively classified as ABCB5 in zebrafish (31) and is an ortholog for ABCB1 and ABCB5 human genes with sequence alignments of zebrafish ABCB5 and human ABCB1 of 57% according to the Ensembl genome database (32).

As reference genes, two housekeeping genes were chosen: EF1α and Rpl13α, since EF1α and Rpl13α genes are validated reference genes for a developmental timecourse study of zebrafish, as well as tissue analysis (33).

Genes' sequences were retrieved from the Ensembl genome database (32). PCR primers and probes for MDR1, as well as EF1α and Rpl13 α, were designed using Universal Probe Library Assay Design Center available on the Web page of Roche applied science (34). Primer pairs for amplifying MDR1/ABCB5 were 5'-CCCCTCCAGAAAAGATGTGA-3' (sense) and 5'-CTGAATCGTGGTGCTTTTCC-3' (antisense).

2.8 cDNA synthesis

0.5 µg of total RNA was reverse transcribed into cDNA using High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. A reverse transcriptase negative control was applied to

test a possible genomic DNA contamination. Obtained cDNA was subsequently diluted 1:40 with RNA-free water for further qRT-PCR performance.

2.9 qRT-PCR

The cDNA was subjected to quantitative PCR analyses using Light cycler 480 probes master mix (Roche, Mannheim, Germany) and the universal probe library with a lightcycler (Roche, England). The final volume of the PCR reaction was 10 µL: 5 µL master mix, 0.1 µL reverse primer, 0.1 µL forward primer, 0.1µL probe, 2.7 µL RNA-free water. All samples were carried out in triplicate in 384-well plates. Dilution series of a mixture of all cDNA from all samples were used as a standard curve. EF1α and Rpl13α were used to normalize the data.

2.10 Expression analysis of ABCB5/MDR1

It has been shown that the use of several reference genes for normalisation of expression levels of target genes instead of one enables more accurate measurement of small expression differences and gives statistically more accurate results (35-36), considering the fact that there is no single reference gene whose expression level is the same in all cell types and under all experimental conditions (37). The geometric mean of reference genes expression was chosen as a specific normalisation factor. Normalized expression levels of MDR1/ABCB5 were calculated by dividing MDR1 values by the geometric mean of two reference genes as described previously by *Vandesompele, 2002* (35).

Statistics

2.11 Statistical analysis

6-7 embryos per well were used to score n=1. Each experiment was repeated at least 3 times over the course of several weeks. To investigate whether the obtained results are statistically significant, a two-tailed paired Student's t-test was used. The level of significance was defined at P-value <0.05. In all graphs the mean values and standard error of the mean (SEM) are shown.

III. Results

3 dpf zebrafish embryos studies

3.1. Dose-response studies

Digitoxin

Following 3 h incubation, digitoxin was shown to cause death and increase the toxicity score with a concentration of 7.5 μM and above in comparison to control. At a concentration of 15 μM, mortality went up to 96%. A digitoxin concentration of 3.75 μM and below did not result in any changes in lethality or toxicity score in comparison with control. (Fig.1,2)

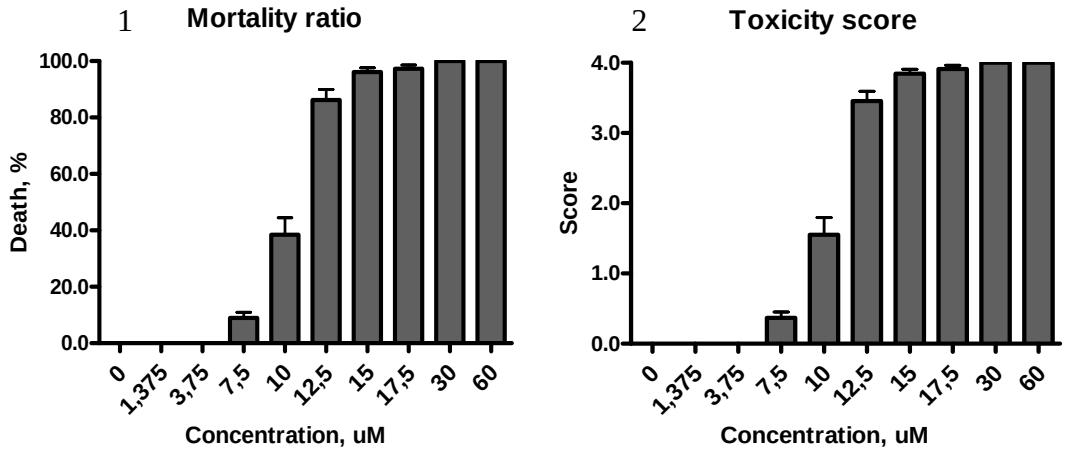

Figure 1, 2. Mortality ratio (1) and toxicity score (2) of 3dpf zebrafish embryos after 3 h incubation with different concentration of digitoxin. The mortality rate is expressed as the percentage of dead embryos to the total amount. Toxicity presented on the second graph was scored using the scoring system described above. Graphs represent the mean values ± SEM of 9-27 samples consisted of 7 embryos for each experimental group.

According to the results, our toxicity score performed similarly to the observed mortality rate: LD50 was calculated to be 10.51 μM and the digitoxin concentration causing a two fold increase in toxicity score was 10.49 μM. These results demonstrated that our scoring system can be reliably used for the toxicity assessment in subsequent experiments on 3dpf zebrafish embryos.

Based on data obtained from these digitoxin dose-response studies, concentrations to be used in subsequent chronotoxicity studies were chosen: 7.5, 10, 12.5, 15 μM.

Verapamil & Ketoconazole

After 1 h incubation, the mortality rate of 10.2% with the concentration of 75 μM verapamil and 12.5% with the concentration of 150 μM ketoconazole was recorded. Higher doses of verapamil that were used in previous studies on 7 dpf zebrafish (25-26) were found to be toxic for 3 dpf embryos resulting in cardiovascular dysfunction and mortality (Fig.3). At concentration of verapamil 50 μM, one death was recorded, which resulted in a 0.53 % mortality in the dose-response study (Fig.3). However, in all following studies neither lethality nor sign of cardiovascular toxicity was demonstrated after 1 h incubation of 3dpf embryos with verapamil. Possibly, this death can be considered as a natural death that might occur due to the stress that fish undergo during incubation in a small volume of water.

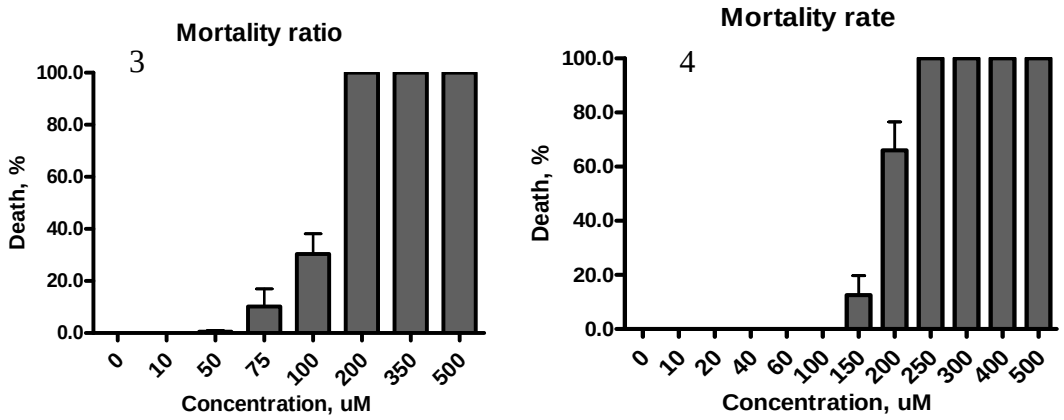

Figure 3, 4. Mortality ratio of 3dpf zebrafish embryos after 1 h incubation with different concentration of verapamil (3) and ketoconazole (4). The mortality rate has been expressed as the percentage of dead embryos to the total amount. Graphs represent the mean values ± SEM of 3-15 samples consisted of 7 embryos for each experimental group.

Accordingly, based on data obtained from verapamil and ketoconazole dose-response studies, maximum non-toxic concentrations which should be used in Pgp inhibitory studies were chosen for both drugs: 50 μM for verapamil and 100 μM for ketoconazole.

3.2 Chronotoxicity studies

To investigate whether there is a diurnal difference in toxicity manifestation, we exposed 3dpf zebrafish embryos to digitoxin at two different time points: at the beginning (ZT 1-4) and at the end (ZT10-13) of their light cycle.

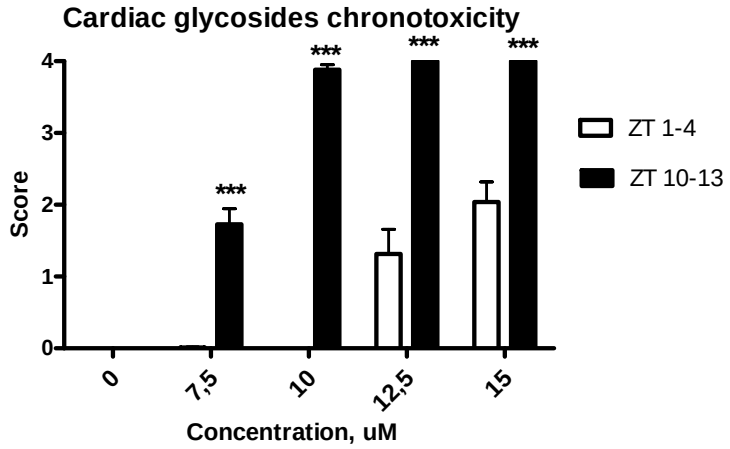

Figure 5. Toxicity score of 3dpf zebrafish embryos after 3 h incubation with different concentrations of digitoxin, scored at two different time points: at the beginning, ZT1-4 (open bars), and at the end, ZT 10-13 (black bars) of the light cycle. The toxicity was scored using the scoring system described above. Graphs represent the mean values ± SEM of 6-38 samples consisting of 7 embryos for each experimental group. ***P < 0.001 by two-tailed paired Student's t-test for comparison of toxicity in each DT concentration group between two different time points: ZT 1-4 and ZT 10-14.

The present study has shown the circadian difference in digitoxin toxicity: the toxicity score was significantly higher (P<0.001) at all tested concentrations at the end of the light cycle (Fig. 5). Remarkably, digitoxin concentrations of 7.5 μM and 10μM resulted in an increase in toxicity score to 1.7 and 3.9 respectively at ZT10-13, while at ZT 1-4 the toxicity score with both concentrations remained around 0.

3.3 Gene expression studies

A noticeable diurnal difference in toxicity demonstrated above (Fig.5) drove us to investigate Pgp gene expression levels. According to the literature, Pgp is not expressed before 5 dpf, as has been mentioned above (25). To confirm these previously reported findings, MDR1/ABCB5 gene expression studies were performed with 3 dpf embryos. Furthermore, to investigate whether there is a diurnal difference in MDR1 expression, samples for qRT-PCR were collected right after incubation at two time points: ZT 4 and ZT 13.

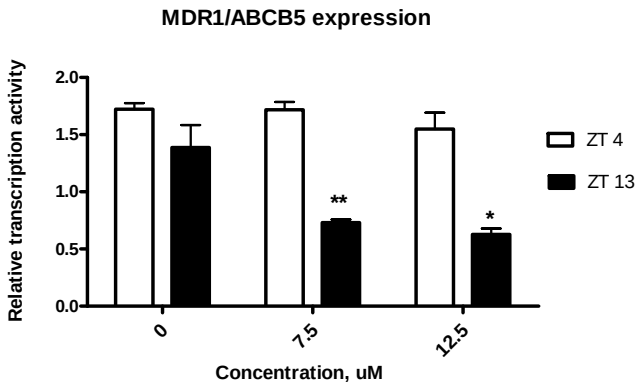

Figure 6. Relative gene expression of MDR1 at the beginning, ZT4 (open bars), and at the end, ZT 13 (black bars) of the light cycle. MDR1/ABCB5 mRNA expression levels were normalized to those of EF1α and Rpl13α. Graphs represent the mean values ± SEM of 3-5 samples consisted of 15 3dpf embryos for each experimental group. $^{*}P<0.05$ $^{**}P<0.01$ by the two-tailed paired Student's t-test for comparison of MDR1 expression in each DT concentration group between two different time points: ZT 4 and ZT 13.

Our results have shown decreased MDR1/ABCB5 expression levels in all experimental groups at ZT 13. However, only in drug-treated groups this decrease was found to be statistically significant, while comparing MDR1 expression levels in each DT-treated group between ZT 4 and ZT 13. (Fig. 6)

Though MDR1/ABCB5 expression levels in the control group at ZT 13 was noticeably lower than at ZT 4, it was not found to be statistically significant due to the high deviation (Fig.6)

3.4. PgpP inhibition studies

The next step was to influence digitoxin toxicity manifestation by known Pgp inhibitors: verapamil and ketoconazole.

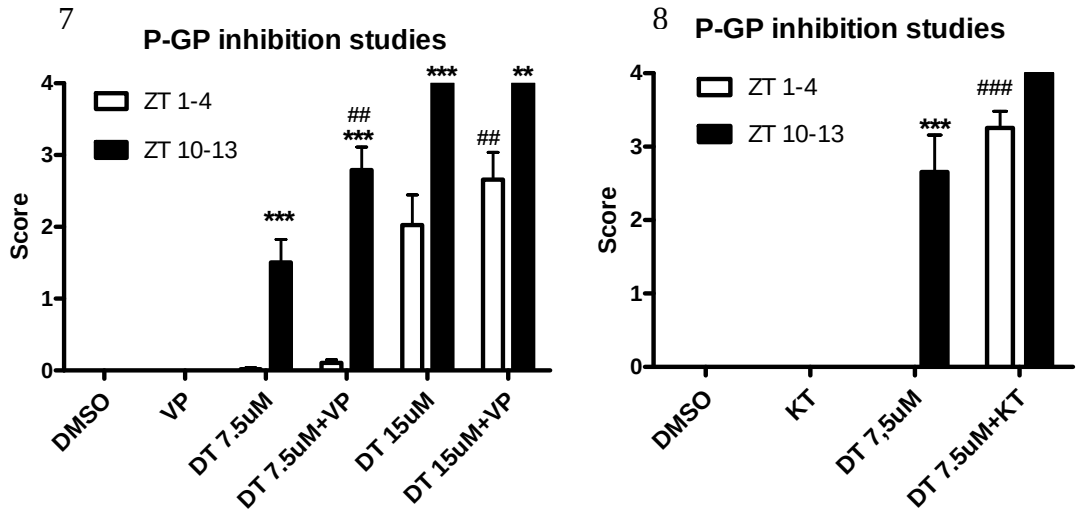

Figure 7, 8. Toxicity score of 3dpf zebrafish embryos after 3 h incubation with two different concentrations of digitoxin with or without preincubation with VP/KT, scored at two different time points: at the beginning, ZT1-4 (open bars), and at the end, ZT 10-13 (black bars) of the light cycle. The toxicity was scored using the scoring system described above. Graphs represent the mean values ± SEM of 4-18 samples consisting of 7 embryos for each experimental group in VP studies and 4-12 samples for KT studies. **P < 0.01 and ***P < 0.001 by two-tailed paired Student's t-test for comparison of toxicity in each DT concentration group between two different time points: ZT 1-4 and ZT 10-14; $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.001 by two-tailed paired Student's t-test for comparison of toxicity in each DT concentration group between DT only treated groups and DT+ VP/KT- treated groups at ZT 1-4 and ZT 10-14 separately.

It has been demonstrated that verapamil pretreatment significantly increased toxicity in the 7.5 μM DT+VP-treated group at ZT 10-13 and in 15 μM DT+VP-treated group at ZT 1-4 in comparison with DT-only treated groups accordingly. Verapamil was shown to increase the toxicity score slightly, though not significantly, in the 7.5 μM DT+VP-treated group at ZT 1-4 versus the 7.5 μM DT-only treated group after 3 h incubation. On the other hand, the difference in toxicity in the DT+VP-treated groups between ZT 1-4 and ZT 10-13 was still statistically significant (P<0.01, P<0.001) even after verapamil pretreatment (Fig.7)

Due to the high toxicity score in the 7.5 μM DT-only treated group at ZT 10-13, a noticeable increase in toxicity (100% mortality) after ketoconazole pretreatment was not found to be significant. Remarkably, in contrast to the results obtained from verapamil studies, ketoconazole significantly (P<0.001) increased toxicity in the 7.5 μM DT+KT- treated group at ZT 1-4. Moreover, no statistically significant difference in

toxicity in the 7.5 μM DT+KT- treated group at ZT 1-4 in comparison with ZT 10-13 was found (Fig.8).

>7 dpf zebrafish studies

3.5. Chronotoxicity studies

Since it has been suggested that Pgp is not expressed before 5 dpf (12,35), we decided to perform chronotoxicity studies and gene expression studies on fish aged above 5 dpf: 8 dpf fish. Zebrafish were exposed to DT [7.5μM] at two different time points: at the beginning (ZT 1-4) and at the end (ZT10-13) of their light cycle.

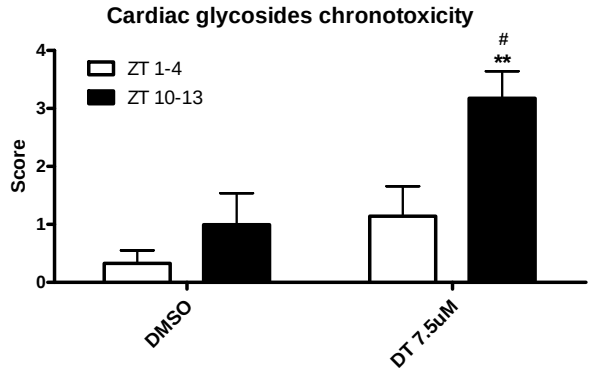

Figure 9. Toxicity score of 8dpf zebrafish fish after 3 h incubation with DMSO or digitoxin [7.5μM], scored at two different time points: at the beginning, ZT1-4 (open bars), and at the end, ZT 10-13 (black bars) of the light cycle. The toxicity was scored using the scoring system described above. The graph represents the mean values ± SEM of 5-6 samples consisting of 6-7 embryos for each experimental group. **P < 0.01 by two-tailed paired Student's t-test for comparison of toxicity in the DT-treated group between two different time points: ZT 1-4 and ZT 10-14; $^{\#}$P < 0.05 for comparison of toxicity in two groups: control and DT-treated group at ZT 1-4 and ZT 10-14 separately.

Accordingly, a significantly higher toxicity in the DT-treated group at ZT 10-13 was demonstrated in comparison with the control group ($P<0.05$) at ZT 10-13, as well as with the DT-treated group at ZT 1-4 ($P<0.01$). These results correlate with our data obtained from 3dpf zebrafish studies, though, in contrast with earlier performed experiments, control groups in studies on 8 dpf have been scored as well due to the application of the new scoring system with different criteria.

3.5. Pgp inhibition studies

Pgp inhibition studies with ketoconazole were performed on 8 dpf fish.

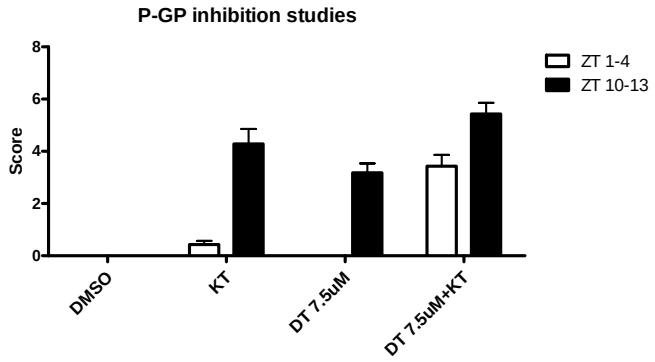

Figure 10. Toxicity score of 8dpf zebrafish embryos after 3 h incubation with digitoxin with or without preincubation with KT, scored at two different time points: at the beginning, ZT1-4 (open bars), and at the end, ZT 10-13 (black bars) of the light cycle. The toxicity was scored using the scoring system described above. Graphs represent the mean values ± SEM of 2 samples consisting of 7 embryos for each experimental group.

Remarkably, in contrast to the results from 3dpf zebrafish studies, a ketoconazole concentration of 70 µM was highly toxic for 8dpf fish at ZT 10-13 and negligibly increased toxicity score at ZT 1-4. Moreover, digitoxin itself augmented the toxicity score up to 3.2 at ZT 10-13, while no toxicity was observed at ZT 1-4. After preincubation for 1 hour with ketoconazole, the toxicity score increased up to 3.5 in the 7.5 µM DT+KT- treated group at ZT 1-4: the same toxicity level as was observed in 7.5 µM DT-only treated group at ZT 10-13. Since ketoconazole was toxic by itself for zebrafish at ZT 10-13, it was not possible to estimate synergistic toxicity in 7.5 µM DT+KT- treated group at ZT 10-13. (Fig.10)

3. 7. Gene expression studies

The present study has demonstrated consistently lower MDR1/ABCB5 expression levels at ZT 13 in comparison with ZT 4, though, just like in studies on 3dpf zebrafish, it was not found to be statistically significant (Fig.11)

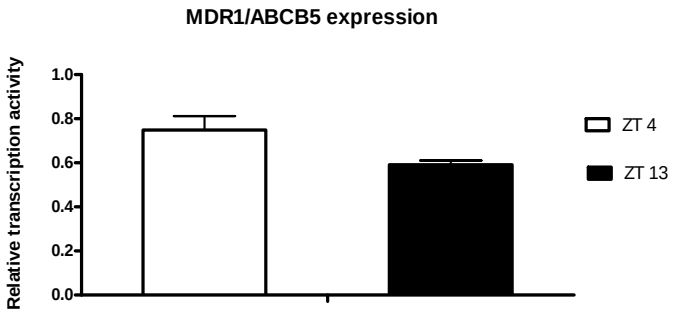

Figure 11. Relative gene expression of MDR1 at the beginning, ZT4 (open bars), and at the end, ZT 13 (black bars) of the light cycle. ABCB5/MDR1 mRNA expression levels were normalized to those of EF1α and Rpl13α. Graphs represent the mean values ± SEM of 3 samples consisting of 10 10dpf embryos for each experimental group.

In the experiments on 8 dpf zebrafish, MDR1 expression levels have been estimated at different time points during the day: ZT 4, ZT 8 and 1 hour before the lights were on, ZT 23. (Fig 12)

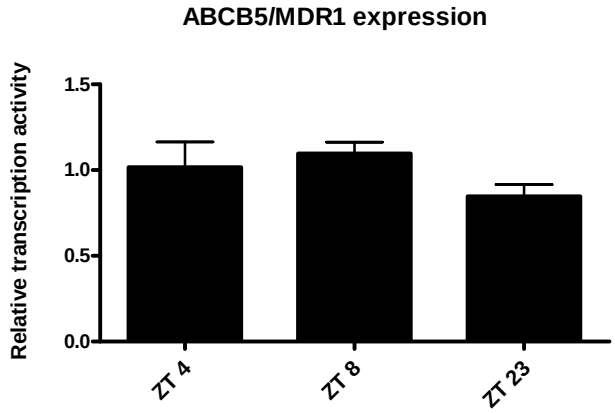

Figure 12. Relative gene expression of MDR1 at different time points of the light cycle: ZT 4, 8, 23. MDR1/ABCB5 mRNA expression levels were normalized to that of EF1α and Rpl13α. Graphs represent the mean values ± SEM of 5-7 samples consisted of 12 8dpf embryos for each experimental group.

Though no statistically significant difference was found between experimental groups, a lower MDR1 expression level at ZT 23 in comparison with ZT 4 and 8 was demonstrated. (Fig.12)

Unfortunately, due to the lack of time and zebrafish embryos, it was not possible to use more time points for gene expression studies and to define an accurate trend in diurnal fluctuation of MDR1 expression levels.

IV. Discussion

Our chronotoxicity studies on 3 and 8 dpf zebrafish embryos unveiled diurnal variation in digitoxin toxicity. We showed a significantly higher toxicity at the end of the light cycle versus the beginning of the light cycle. Though it was shown that toxicity, caused by the lowest concentration of digitoxin used in this study, increased significantly after verapamil pretreatment at ZT 10-13, no augmentation in toxicity was shown at ZT 1-4. Remarkably, significant increase in digitoxin toxicity was observed after ketoconazole pretreatment at ZT 1-4 till the toxicity level demonstrated at ZT 10-13. However, no statistically significant difference in diurnal variation in MDR1 expression levels was demonstrated in this study neither in 3, 8 nor 10 dpf zebrafish.

Nevertheless, the question may arise whether the circadian difference in toxicity manifestation in 3 dpf embryos is related to the difference in developmental stage, since the age difference of embryos at the beginning and at the end of the light cycle could constitute 5-8 hours. For example, it has been shown that CYP3A enzymes, that comprise the largest part of the liver and small intestine enzymes and are responsible for activation and inactivation of numerous endogenous and exogenous compounds, differ in their expression levels during zebrafish development (44-45). Thus, zebrafish CYP3A65 (ortholog to human CYP3A4) has been shown to appear in a zebrafish embryo's liver and proximal intestine at 72 hpf with a considerably increased expression after 84 hpf due to maturation of the gut and yolk resorption (44). On the other hand, a high CYP3A level cannot explain higher toxicity of digitoxin at 80-82 hpf in comparison with 72-73 hpf, because in this case we would expect a higher metabolism rate with a reduction in toxicity manifestation.

Though expression of CYP3A does not seem to be involved in observed diurnal difference in toxicity in 3dpf zebrafish embryos, there are other numerous factors which could possibly be involved, such as a proceeding functional maturation of different organs, increased capability of gas exchange, etc.

Therefore, taking into account the possible influence of an active early developmental process, as well as the previously suggested hypothesis that Pgp is not expressed in zebrafish before 5 dpf, digitoxin chronotoxicty studies have been repeated on 8 dpf fish. Our results from these studies were in line with above mentioned studies on 3 dpf embryos: significantly higher toxicity was demonstrated at ZT 10-13 in comparison with ZT 1-4.

In this study the increased toxicity of digitoxin with verapamil has not been revealed in all experimental groups. Though verapamil significantly increased the toxicity in VP+DT7.5μM at ZT 10-13 and VP+DT15μM at ZT 1-4, we did not observe the increase in VP+DT7.5μM at ZT 1-4 till the toxicity level demonstrated in this group at ZT 10-13. This observation is possibly due to the low dose of verapamil used in the present study. It was reported previously that verapamil effectively inhibits Pgp in the blood brain barrier (BBB) in zebrafish at a concentration of 100 μM (25-26). Since we observed cardiac toxic effects with this dose on 3 dpf zebrafish embryos, a two times lower concentration of 50μM was used in our study.

Furthermore, recent studies on Pgp-expressing cell lines showed that the Pgp inhibitory potency of verapamil metabolites is higher than that of a parent compound: IC_{50} of verapamile metabolite nonverapamil was shown to be ~4 times higher than VP IC_{50} (42). Since CYP3A expression is lower in 3dpf embryos in comparison with later stage of zebrafish development, as has been mentioned before (44), it has therefore been suggested that lower rate of verapamil metabolism and active metabolites formation in 3 dpf zebrafish embryos might contribute to the poor Pgp-inhibitory activity of verapamil and as a result, absence of supra-additive toxicity of VP+DT7.5μM in the present study on 3dpf embryos.

Therefore, more potent pharmacological Pgp inhibitor ketoconazole with no known cardiac toxicity has been used in the following studies (39). Significant augmented toxicity in KT+DT 7.5μM-treated group has been demonstrated at ZT 1-4: toxicity score increased substantially in KT+DT 7.5μM-treated group, while no toxicity has been observed in KT or DT-only treated groups at ZT 1-4. Remarkably, toxicity in KT+DT 7.5μM -treated group at ZT 1-4 reached the level detected at DT-only treated group at ZT 10-13.

Inhibition of Pgp and, as a result, increase in digitoxin bioviability could possibly explain observed phenomena. However, aside KT's inhibitory effect on Pgp, it is known for its CYP3A inhibition activity (49). Therefore, our results are limited by possible alteration of metabolism of digitoxin due to the CYP3A inhibition by ketoconazole. Though there is compelling evidence that digitoxin undergoes liver metabolism, the rate at which this metabolism occurs is still disputed (50-51). In several *in vivo* animal studies, it was suggested that digitoxin is largely metabolised by liver microsomes to active, as well as inactive metabolites (50). However, in later

conducted studies, the species differences in digitoxin metabolism and toxicity has been revealed. Accordingly, it was demonstrated that the overall rate of digitoxin oxidation was lowest in human in comparison with eight other mammalian species, including rat, which has usually been used for digitoxin metabolism studies (51). Previously, it has been reported that up to 48% of digitoxin is eliminated intact in urine and faeces in human (52). Moreover, it is well known that digitoxin is excreted slowly over a period of several days (50,52).

Thereby, taking into account the short period of incubation and a relatively high amount of drug in the incubation medium, the metabolism of digitoxin might not have a large impact on toxicity manifestation. Nevertheless, the possibility, that the observed synergistic toxicity in KT+DT7.5µM-treated group is partly due to the ketoconazole-induced inhibition of CYP3A and decrease of the metabolism of digitoxin in zebrafish, cannot be ruled out.

This is further emphasised by Pgp inhibition studies on 8 dpf fish. Like in Pgp-inhibition studies on 3 dpf fish, increased toxicity has been demonstrated in KT+DT 7.5µM-treated group at ZT 1-4. Moreover, toxicity in this group reached the level of toxicity observed in DT-only treated group at ZT 10-13. Remarkably, ketoconazole was highly toxic at the dose of 100µM for 8 dpf zebrafish, while no toxicity was observed in 3dpf embryos. This phenomenon might be explained by higher expression of CYP3A in 8 dpf versus 3 dpf fish (44). In several studies it was suggested that metabolites of ketoconazole, e.g. N-deacetyl ketoconazole (DAK), are more toxic than the parent compound itself (53-54). Accordingly, the higher ketoconazole metabolism rate in 8dpf fish may result in an increase of toxic metabolites formation and, as a result, toxicity manifestation. It was decided to reduce the ketoconazole dose to 70 µM for Pgp inhibition studies on 8 dpf fish. Interestingly, this dose of ketoconazole was still highly toxic for zebrafish at ZT 10-13, while at ZT 1-4 the increase in the toxicity score was neglectable. Since ketoconazole is a substrate for Pgp itself, circadian differences in Pgp activity could be responsible for the observed differences in toxicity at the beginning and at the end of the light cycle. Thus, it was reported, that itraconazole (which is an azole antifungal drug similar to ketoconazole) undergoes Pgp efflux in BBB (55).

It is possible that not only circadian differences in Pgp expression, but also CYP3A expression might have an impact on drug toxicity. This is supported by recent studies, which showed that both mRNA levels and metabolic activity of CYP3A4 in serum-

shocked HepG2 cells fluctuated during the day (56). Additionally, 6β-OHC/C ratio in human, which is considered to reflect CYP3A activity, varied during the day with a reduced ratio in the morning versus evening hours (47). Unfortunately, there is no data available regarding the diurnal variation in Pgp expression in human. However, the observed variation in CYP3A expression in human during the day correlates with Pgp expression showed in mouse models: higher CYP3A activity at the end of the light cycle corresponds with higher Pgp expression levels (7,47).

Remarkably, MDR1 expression levels were consistently, thought not significantly, lower at the end of the light cycle both in 3 and 10 dpf fish. Moreover, gene expression studies on 8 dpf fish revealed slightly lower MDR1 expression levels one hour before the light went on (ZT 23) in comparison with ZT 4 and ZT 8. These data suggest that MDR1/ABCB5 expression levels go up slightly at the beginning of the light cycle, followed up by a decline at the end of a light cycle and beginning of a dark phase. However, due to the small numbers of samples it was not possible to detect significant diurnal differences in MDR1 expression levels. Also, measurement of MDR1 expression at other different time points throughout the day, would add reliability to our results.

Interestingly, MDR1 expression levels dropped significantly following 7.5 and 12.5 µM DT treatment at ZT 10-13, while at ZT 1-4 no changes in transcription activity were demonstrated. Since in 7.5 and 12.5 µM DT-treated groups mortality was observed, it could be argued that the downregulation of MDR1 expression was caused by RNA degradation and loss of integrity. However, this suggestion can be refuted, as extracted RNA levels of both housekeeping genes and MDR1 was high. Also, transcription activity of EF1α and Rpl13 α were at normal levels in all experimental groups, ruling out the possibility of low MDR1 levels in drug-treated groups due to RNA degradation. Moreover, it has been demonstrated in different time-course degradation studies that no loss of RNA integrity or decrease in gene expression levels occur in breast tissue up to 3 hours and in lung tissue up to 5 h after surgical removal. Nevertheless, mRNA degradation seems to depend on the type of cells and size of the mRNA, which makes these findings questionable (46).

Poor Pgp efflux of digitoxin at the end of a light cycle could result in a higher digitoxin bioavailability and, as a result, greater impact on gene expression levels. Though, the observed slight difference in diurnal MDR1 expression levels in control groups cannot fully explain the considerable difference in MDR1 expression levels in DT-treated

groups at ZT 10-13 and ZT 1-4. On the other hand, the chosen ABCB5 gene might not be fully representative of the entire MDR1 gene and, moreover, gene transcription activity does not always correlate with protein expression levels, which were not measured in the present study. Moreover, it was suggested that another mechanism, such as enzyme inhibition or allosteric effects by physiological regulators might be responsible for circadian changes of enzymes, e.g. CYP3A (47). Therefore, gene or protein expression levels might not always represent the actual activity of transporters or enzymes; and specific functional assays should be performed to assess diurnal changes in their activity.

Based on results obtained from an *in vitro* study with human intestinal epithelial cells, it was suggested that chronic pre-treatment with low doses of digitalis cardiac glycoside digoxin upregulates MDR1 expression (48). On the other hand, downregulation of MDR1 by digitoxin was shown in the present study. Therefore, it appears that acute toxic exposure might affect MDR1 expression differently through other molecular mechanisms. Moreover, the fact that digitoxin acts differently from digoxin cannot be ruled out either. Also, to date no complete coding sequence of a fish MDR1 gene is available, limiting to some extent pharmacological and toxicological studies aimed at identifying substrates and modulators of the Pgp transporter (41).

Remarkably, in zebrafish we observed diurnal variation in cardiac glycoside toxicity opposite to that of human: with higher toxicity at the end versus the beginning of the light cycle. These observations lead us to the conclusion that the circadian variation in enzymes and transporters activity might differ between species, something that should be taken into account in pharmacological and toxicity studies.

Finally, the present study demonstrated that zebrafish can be an excellent model for cardiac glycosides chronotoxicity studies. In previous studies it has been shown that zebrafish heart cells contain sodium, potassium and calcium channels which possess conductivity similar to those in other species (15). That makes zebrafish an excellent model for cardiac glycosides toxicity studies in particular, due the fact that these compounds act through changes in the Na^+/K^+ channels function (11).

Furthermore, in the present study, new reproducible toxicity scoring systems have been developed to assess toxicity in 3 and 8dpf fish accordingly. Two scoring systems proved to be reproducible over the course of numerous studies. Although, assessment of other criteria, such as ejection fraction, motility or precise evaluation of blood flow

velocity (μm/sec) and heart beat (b/min), could add more accuracy to the toxicity scoring system for 3 dpf embryos, it was not feasible in this particular study due to the limitation of time within which the toxicity should be evaluated. Estimation of cardiac function in 8dpf zebrafish is limited by initiation of pigmentation of the fish. Therefore, phenotypical toxicity criteria were included to the scoring system for 8 dpf, such as response to stimuli and body position, that in turn resulted in a low toxicity score in the control groups as well. This can be explained due to the stress that fish might endure during incubation in a low volume of water.

In summary, the present study highlights the suitability of zebrafish as a model to perform cardiac glycosides chronotoxicological studies, since a significant diurnal difference in digitoxin toxicity has been demonstrated on 3 and 8 dpf zebrafish. Although the diurnal difference in MDR1 expression between ZT 4 and ZT 13 was not found to be significant due to the low numbers of samples, the general fluctuation trend in MDR1 expression levels corresponded well with variation in toxicity manifestation at the beginning versus the end of the light cycle. Also, gene expression levels not always represent actual activity of the transporter. Additionally, ketoconazole has been shown to increase DT toxicity. However, the observed synergistic toxicity of DT with KT could be explained by inhibition of both Pgp and CYP3A, which cannot be differentiated in the present study.

Limitations of this study, such as early stage of zebrafish development and non-selectivity of Pgp inhibitors used in experiments, make it impossible to draw a definite conclusion regarding the involvement of Pgp in cardiac glycosides chronotoxicity. Pgp antibody staining and performance of functional Pgp activity tests with fluorescent compounds such as rhodamine 123 would provide support to the initial hypothesis. In spite of these restrictions, the present study suggests that the role of Pgp might play an important but not exclusive role in cardiac glycosides chronotoxicity and another factors, such as liver enzymes activity (CYP3A in particular) should be taken into consideration. Therefore, it is unlikely that one mechanism is responsible for all chronopharmacological phenomena and different factors can have an impact on the outcome of the drug therapy.

In conclusion, our study provided evidence to support the validity of a zebrafish model to study chronopharmacological phenomena. Although there are still many unresolved questions and areas of controversy, our results contribute to the hypothesis of possible Pgp involvement in cardiac glycosides chronotoxicity. Nevertheless, more studies

need to be performed to unveil the actual contribution of Pgp in observed diurnal changes in cardiac glycosides toxicity.

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