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Functional studies on the activity of efflux transporters in an ex vivo model with chicken splenocytes and evaluation of selected fluoroquinolones in this model

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ABSTRACT

The efflux proteins P-glycoprotein (P-gp), BCRP and members of the MRP-family (MRPs) are increasingly recognized as determinants of the absorption, tissue distribution and excretion of numerous drugs. A widely applied in vitro screening method, to assess the effect of these efflux transporters in transmembrane transport of drugs is based on the use of peripheral blood mononuclear cells (PBMC), in which the efflux of fluorescent dye Rhodamine 123 (Rh-123) can be easily measured. In avian species, the isolation of PBMCs is compromised by the presence of thrombocytes having approximately the same size. As an alternative, we validated the use of isolated splenocytes to assess Rhodamine 123 transport in the presence and absence of specific inhibitors for P-gp, MRPs and BCRP. Rh-123 efflux was concentration-dependent with the percentage of efflux that decreased with increasing concentrations. P-gp inhibitors, PSC833 and GF120918, significantly inhibit Rh-123 efflux, whereas inhibitors for MRPs and BCRP, MK571 and Ko-143, respectively, have a limited inhibitory effect. However, the effect of GF120918 was more pronounced as compared to PSC833, suggesting an additional role for BCRP next to P-gp in Rh-123 efflux. Moreover, fluoroquinolones were selected to test the applicability of the described model. None of these fluoroquinolones significantly inhibit P-gp function at concentrations up to 50 μM, with exception of danofloxacin and danofloxacin mesylate that were found to reduce Rh-123 efflux by approximately 15%.

1. Introduction

Members of the ATP-Binding Cassette (ABC), a large superfamily of transmembrane proteins that use the energy of ATP hydrolysis to transport molecules across cell membranes [40], have been recognized as cellular efflux transporters for drugs and toxins, with P-glycoprotein (P-gp) (ABCB1) being the most widely studied. P-gp has a broad substrate specificity and is

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Abbreviations: ABC, ATP-Binding Cassette; BCRP, breast cancer resistance protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MDR1, multiple drug resistance 1; MRP2, multidrug resistance-associated protein 2; PBMC, peripheral blood mononuclear cells; P-gp, P-glycoprotein; Rh-123, Rhodamine 123

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widely distributed in different tissues in mammals and in poultry [4,15,18]. Other widely distributed ABC-transporters that transport drugs and toxins are BCRP (ABCG2) and MRP1 (ABCC1) [26], but their homologues have not been studied in poultry species yet. In addition, mammalian MRP2 is a transporter of mainly conjugates of endogenous and exogenous compounds and recently we showed its expression in chickens with high levels in the liver, kidneys and upper intestinal tract [18]. Several other members of the MRP-family transport drugs and conjugates, but these have a limited tissue distribution in mammals [12], and have not been studied in poultry.

In the evaluation of pharmacokinetic properties of new and existing drugs, it is essential to identify whether or not a given molecule is a substrate for one or more transporters, as the latter may restrict the oral availability, determine transport across biological barriers, and influence the routes of excretion [8,21,36,49]. The activity of these ABC-transporters, however, varies among animal species and between individuals. Their transcription is linked to physiological processes, among others the hormonal status of an animal, environmental factors, and feed materials that may contain (competitive) inhibitors [2,3,48,51].

To identify drugs that are substrates for ABC-transporters and to monitor changes in expression and function during treatment, a lymphocyte based ex vivo model has been widely used [1,7,25,34,43]. In these studies, the lipophilic, cationic fluorescent dye Rhodamine 123 (Rh-123) is used as a prototypical P-gp substrate, allowing a simple detection of intracellular Rh-123 retention by FACS analysis and hence the effect of various compounds hereon.

As peripheral blood mononuclear cells (PBMCs) are difficult to isolate as a pure fraction from avian species due to the fact that avian thrombocytes have approximately the same size, we used a comparable model of isolated splenocytes from healthy chickens. To validate this model, the effect of various typical inhibitors for individual ABC-transporters on Rh-123 retention was measured. Finally, the practicability of the model was demonstrated in measuring the potential inhibitory effect of commonly used fluoroquinolones in the same model. Fluoroquinolone antimicrobials have been found to be substrates for one or multiple ABC-drug transporters in mammalian species [22,33,38,39,41] before and members of this class of drugs potentially modulate ABC dependent transport [9,29,42,54,55].

2. Material and methods

2.1. Chemicals and drugs

Rhodamine 123 and cyclosporin A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PSC833 (SDZ 215-833) was kindly provided by Novartis Pharma AG (Basel, Switzerland). GF120918 was kindly provided by Glaxo SmithKline (Stevenage, Herts, UK). Ko-143 was kindly provided by Prof. Koomen (University of Amsterdam, The Netherlands). MK571 sodium salt was obtained from Alexis Biochemicals (San Diego, CA, USA).

Danofloxacin mesylate was kindly provided by Pfizer (Sandwich, Kent, UK). Enrofloxacin and danofloxacin was from Sigma–Aldrich Chemie and marbofloxacin (Marbocyl FD 1%, powder) was from Vetoquinol (France) and ciprofloxacin (Ciproxin 200) was from Bayer (Leverkusen, Germany). All other chemicals were of the highest grade available.

2.2. Animals

Twenty-five hens were included in the experiments. The birds were layers of the breed Bovans Goldline and about 27 months old. They were obtained from a commercial farm (Haasdrecht, The Netherlands) and were housed in a group (at 20 °C) 2 weeks before the start of the experiments in the animal unit of the Faculty of Veterinary Medicine, Utrecht University. Standard commercial feed (without antibiotics and coccidiostats) and water were supplied ad libitum. The use of these animals as organ donors was approved by the Ethical Committee of the Veterinary Faculty.

2.3. Isolation of splenocytes

Spleens were collected immediately after euthanasia of birds and transported in ice-cold Hanks balanced salt solution (HBSS, Gibco BRL, Breda, The Netherlands) to the lab facilities. After disruption of the spleen capsule, a cell suspension was obtained by carefully flushing the spleen-pulp through BD Falcon Cell Strainers (70 μm, no. 352350). Splenocytes were then separated on a Ficoll (Ficoll Paque® Plus Research, Pharmacia Biotech, Uppsala, Sweden) density gradient by centrifugation for 20 min at 500 × *g* at room temperature 22 °C. Interphase cells were collected and washed twice with phenol red-free RPMI 1640 complete medium (Gibco, Grand Island, NY, USA) supplemented with 10% foetal calf serum Gibco (Grand Island, NY, USA) and 1% glutamine. After the second washing step, the cell pellets were re-suspended in Erylysis buffer containing 1% bovine serum albumin (BSA), 0.155 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM disodium EDTA in distilled water, pH 7.2. After 2 min, PBS was added and the sample centrifuged at 267 × *g* for 5 min. The obtained cell pellets were re-suspended in 5 ml supplemented RPMI 1640. The cell suspension was plated into petri dishes and incubated for 1 h at 41 °C in a 5% CO_2 humidified atmosphere to increase the purity by removing the attached macrophages. The cell suspension was collected and stored overnight at 4 °C. Before the re-start of the experiments, the cell suspensions were incubated again at 41 °C and the cell concentrations estimated by counting an aliquot in Turk solution using a haemocytometer.

2.4. Characterization of lymphocyte-subsets obtained from spleen samples

Cell-samples (0.3×10^6 cells) were incubated with the fluorochrome labelled mouse anti-chicken antibodies (diluted 1:150 in FACS buffer consisting PBS with 1% BSA and 0.5% sodium azide), CD3-FITC (8200-02), CD4-FITC (8210-02), CD4-RPE (8210-09), CD8α-RPE (8220-09) and CD8β-RPE (8280-09) (Southern Biotechnology Associates Inc., USA), for 30 min at 4 °C in the dark. The cells were subsequently washed twice with FACS buffer, centrifuged for 5 min at 267 × *g* and re-suspended in FACS buffer. The cell-associated fluorescence

was than measured and quantified by flow cytometry on a FACScalibur fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany).

2.5. Measurement of Rhodamine 123 efflux

Splenocytes suspensions were aliquoted into the wells of a 96 well plate (5×10^4 cells/well), centrifuged ($267 \times g$/5 min) and the supernatant discarded. Cells were then re-suspended and incubated in serum-free, phenol red-free RPMI 1640 medium with Rh-123 at concentrations of 0.063, 0.125, 0.25, 0.5, 1, 2 and 4 μM for 30 min at 41 °C in a 5% CO_2 humidified atmosphere. Control samples were incubated with medium only. Subsequently, cells were washed with ice-cold phosphate buffered saline (PBS) and incubated in dye free, serum-free, phenol red-free RPMI 1640 medium with or without typical inhibitors or fluoroquinolones to allow Rh-123 efflux. The incubation periods were 0, 0.25, 0.5, 1 and 2 h. Various concentrations of typical inhibitors and fluoroquinolones were tested. Final concentrations for the typical inhibitors were: cyclosporin A: 4 μM and 20 μM; PSC833: 1 μM and 5 μM; GF120918: 0.04 μM and 4 μM; MK571: 1 μM and 25 μM; and Ko-143: 0.04 μM and 1 μM. The final concentrations for the fluoroquinolones were 1.56 μM, 3.12 μM, 6.25 μM and 50 μM. In all experiments with fluoroquinolones, GF120918 was included as positive control at a concentration of 4 μM. The final concentration of DMSO, which served as solvent for the drugs as well as the inhibitors was 0.1%, and this concentration was added also to all control samples.

Cell-associated fluorescence was quantified in a FACScalibur fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany) equipped with an argon 488 nm laser. On the basis of forward and side light scatter, lymphocytes were gated and the data were acquired for a total of 10,000 gated cells per sample after exclusion of the cells that were stained positive with propidium iodide (1 μg/ml) as the latter indicates dead and damaged cells. Rh-123 fluorescence was assessed using a 530-nm bandpass filter and propidium iodide fluorescence was measured using a 610-nm bandpass filter. The Rh-123 efflux was calculated from the median Rh-123 fluorescence for each sample at the indicated time-points and was used for the calculation of inhibition by the typical inhibitors or fluoroquinolones. Data acquisition was performed using the computer program CellQuest (Becton Dickinson, Heidelberg, Germany).

The percentage of Rh-123 efflux was calculated as follows:

$$\text{Efflux}_{(t)}\,(\%) = 100 \times \frac{[\text{mFl}_0 - \text{mFl}_{(t)}]}{\text{mFl}_0}, \quad (1)$$

$\text{mFl}_{(t)}$ and mFl_0 represent the median fluorescence, as the obtained data do not follow a Gaussian distribution. Alternatively, the geometric mean of the fluorescence at peak levels could be used for the comparison of data, i.e. the estimation of Rh-123 efflux at time t and time 0, respectively.

The percentage of Rh-123 efflux in the presence and absence of typical inhibitors or the selected fluoroquinolones was plotted as a function of time between 0 and 2 h, and the corresponding areas under the curve (AUC Inhibitor and AUC Control, respectively) were calculated.

The percentage of inhibition was then estimated according to the following equation:

$$\text{Inhibition}\,(\%) = 100 \times \left(\frac{1 - \text{AUC Inhibitor}}{\text{AUC Control}}\right). \quad (2)$$

2.6. RT-PCR analysis

Total RNA was isolated using Trizol Reagent (Invitrogen Life Technologies, Cat no. 15596-018) according to the manufactures instructions. First-strand cDNA was synthesized from 1 μg total RNA with the iScript^{tm}cDNA Synthesis Kit (Bio-Rad Laboratories, USA) in a final volume of 20 μl. The reaction mixture was incubated for 5 min at 25 °C, at 42 °C for 45 min, followed by heat inactivation of the enzyme at 85 °C for 5 min and hold at 4 °C. The obtained cDNA samples were stored at −20 °C.

The development of primers specific for the chicken homologues of MDR1, MRP2 and BCRP and the internal control genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin have been described previously [18] and were commercially produced (Isogen Bioscience BV, The Netherlands) (for details see Table 1). For the PCR reaction, the iQTM SYBR Green Supermix (Bio-Rad Laboratories Inc., USA) was used according to producers' instructions containing 1 μl of reverse transcribed RNA in a reaction volume of 25 μl that was run in a MyIQ single-color real-time PCR detection system (Bio-Rad, Hercules, CA) for 40 thermal cycles. All analyses were performed in duplicate.

2.7. Statistical analysis

The results are presented as mean ± S.D. All data were analysed with the Statistica 6.1 computer program (Statistica for Windows, StatSoft Inc., USA, 1984–2002). Statistical analysis was done by using a one-way ANOVA to assess differences in efflux of various Rh-123 concentrations and in the absence and presence of typical inhibitors or fluoroquinolones. Bonferonni's test was applied as a post hoc test for multiple comparisons between treatments.

Table 1 – Primers used in the PCR

Gene	Forward primer 5′ → 3′	Reverse primer 5′ → 3′
MDR1	GCTGTTGTATTTCCTG CTATGG	ACAAACAAGTGGGCT GCTG
MRP2	CTGCAGCAAAATGAG AGGACAATG	CAGAAGCGCAGAGAAG AAGACCAC
BCRP	CCTACTTCCTGGCCTT GATGT	TCGGCCTGCTATAGCT TGAAATC
GAPDH	GTGTGCCAACCCCCAA TGTCTCT	GCAGCAGCCTTCACTA CCCTCT
Beta-actin	ATGTGGATCAGCAAGC AGGAGTA	TTTATGCGCATT-TATGGGTTTTGT

The nucleotide sequences of the PCR primers used to assay gene expression by real-time quantitative PCR.

3. Results

3.1. Characterization of lymphocytes, isolated from the spleen of healthy chickens

The following subsets of lymphocytes and their relative abundance were detected in the cell-samples derived from the spleen: $CD3^+$: 52.08%, $CD4^+$: 18.7%, $CD8\alpha^+$: 37.6% and $CD8\beta^+$: 29.3%. Some of the cells in the studied populations were double positive ($CD3^+CD8\alpha/\beta^+$ or $CD4^+CD8\alpha/\beta^+$, data not shown), but taken together these data confirm that the used cell population represented lymphocytes.

3.2. mRNA levels of expression of MDR1, MRP2 and BCRP in chicken lymphocytes, isolated from spleen

The expression of the ABC-transporters P-gp, MRP2 and BCRP was confirmed in chicken splenocytes. The Threshold Cycle (CT) values were lowest for P-gp, indicating a higher expression than the other transporters.

3.3. Rhodamine 123 concentration-dependent fluorescence intensity in chicken's splenocytes

After the incubation of splenocytes with increasing concentrations of Rh-123, two peaks with different fluorescence intensity were detected, indicating two subpopulations that differ in Rh-123 uptake (Fig. 1). The fluorescence intensity increased with increasing concentrations of Rh-123 in both subpopulations.

The subpopulation denoted in Fig. 1 as M1 exhibited a high percentage of Rh-123 efflux in the 2 h following the loading period. In contrast, the subpopulation denoted as M2 in Fig. 1 continued the uptake of Rh-123, presumably the Rh-123 that had been effluxed by the subpopulation M1. These findings suggest a slow rate of uptake and a limited efflux capability of the M2 cells. These two populations could not be distinguished, however, on the basis of the results obtained with a forward-versus-side scatter. For subpopulation M1, a concentration-dependent efflux was observed, with an apparent decrease in efflux rate at higher concentrations of Rh-123 (Table 2) indicating saturation in the transport.

3.4. Effect of proto-typical ABC inhibitors on Rhodamine 123 efflux

The effect of typical inhibitors for P-gp, MRP's and BCRP on Rh-123 efflux was evaluated following the incubation of samples with increasing Rh-123 concentrations and measuring subsequently the efflux in dye free medium in the presence or absence of two different concentrations for each individual inhibitor. A limited, but highly variable inhibition was found for the samples that had been incubated with 1 μM, 2 μM and 4 μM Rh-123 and hence only the lower Rh-123 concentrations were used in the forthcoming experiments. A significant inhibition of Rh-123 efflux (at Rh-123 concentrations of

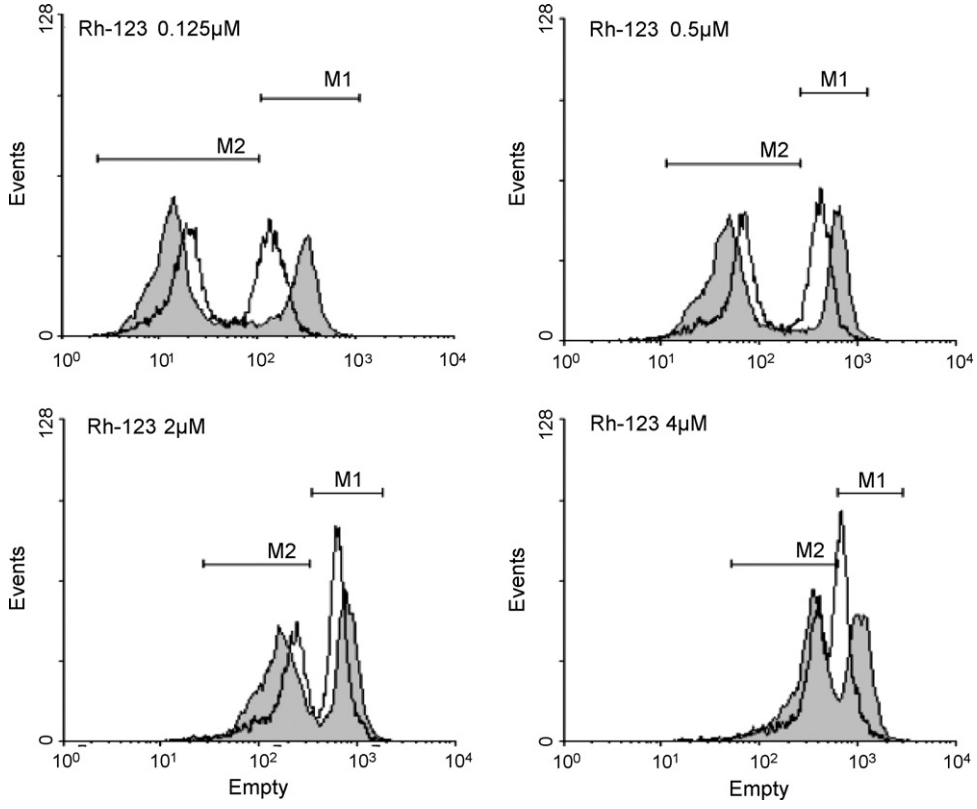

Fig. 1 – Representative histograms of Rh-123 retention in lymphocytes: two peaks were detected representing two subpopulations of cells (peak M1 and M2). Filled area represents cellular Rh-123 intensity at t = 0 h and the unfilled area represents the intensity after 2 h of incubation in dye free medium. *X*-axis: fluorescence intensity of Rh-123 (four-decade logarithmic scale). *Y*-axis shows the number of events (arithmetic scale).

Table 2 – Concentration-dependent efflux of Rhodamine 123 (Rh-123) from lymphocytes (region M1), mean ± S.D. of five experiments

Concentration of Rh-123 (μM)	Efflux of Rh-123 (%)
0.0625	59.69 ± 7.50
0.125	56.20 ± 3.82
0.25	47.73 ± 6.87
0.5	31.21 ± 3.60
1	21.19 ± 7.48
2	19.07 ± 8.31
4	25.40 ± 7.33

0.0625 μM and 0.5 μM) was observed in the presence of GF120918 (0.4 μM and 4 μM) and PSC833 (1 μM and 5 μM) (*P*-values of < 0.001–0.04) (Fig. 2). The most pronounced inhibitory effect was observed when the samples were incubated with 4 μM GF120918, ranging from 68.13 ± 6.49 to 84.45 ± 9.71% when lymphocytes were incubated with Rh-123 concentrations between 0.0625 μM and 0.5 μM (*P*-values of <0.003). Cyclosporin A at concentrations of 4 μM significantly inhibits Rh-123 efflux applied at a concentration of 0.5 μM (*P*-value of 0.001). The higher concentration of cyclosporin A and the investigated concentrations of MK571 did not change significantly the efflux of the fluorescent dye. Inhibition of Rh-123 efflux was observed in the presence of 1 μM Ko143.

3.5. *Effect of fluoroquinolones on Rhodamine 123 efflux*

The inhibitory effect of the fluoroquinolones on Rh-123 efflux was evaluated with samples that were incubated with 0.125 μM and 0.5 μM Rh-123 and data were only interpreted for the population M1. Ciprofloxacin, marbofloxacin and enrofloxacin did not exert any effect on Rh-123 efflux. Danofloxacin mesylate decreased Rh-123 efflux when applied at concentrations of 3.12 μM and 50 μM in samples incubated with 0.5 μM Rh-123, while no effect was observed for the samples incubated with 0.125 μM Rh-123 (Table 3).

4. Discussion

Various cell types, including peripheral mononuclear cells, are known to express ABC efflux transporters. Hence, isolated cells and cell cultures might serve as elegant models in screening of drug-transporter interaction at the functional level and can be applied both, in the phase of drug development as well as in the in vivo/ex vivo monitoring of the potential effects of drugs and toxins on the level of expression of these efflux transporters. In previous experiments, mainly peripheral mononuclear cells have been used, as these can be obtained easily from blood samples. However, in smaller animals, the number of obtainable lymphocytes is limited, and often does not allow to conduct a full set of experiments with cells from one individual. Moreover, substantial differences between mammals and chickens in the morphology of blood cells have been described showing that avian thrombocytes have the same size as small lymphocytes [24,30,31,37]. Hence, even the separation of blood samples from chickens over a Ficoll-Paque density gradient results in a cell fraction that contains up to 60–70% thrombocytes [6,23]. This fact impairs the use of lymphocytes as an easy model for drug transport experiments, and hence we used splenocytes from healthy donor animals as surrogates for peripheral blood mononuclear cells. Previous experiments had demonstrated that these splenocyte fractions contain at maximum 5% thrombocytes [6,23,24]. The homogeneity of the obtained cell population was confirmed by positive staining with various antibodies, indicating that $CD3^+$ and $CD8\alpha^+$ cells were the most predominant cell types, followed by $CD8\beta^+$ and $CD4^+$ cells. In human lymphocyte-subsets, previously used to investigate the function of ABC-transporters, as for example P-gp expression and activity, the dominant cell types were also CD4+, CD8+ and CD56+ cells [5,14,43].

The function of efflux transporters in isolated splenocytes was estimated by measuring the rate of efflux of the fluorescent dye Rh-123. Cells were first loaded with Rh-123 and then placed into fresh medium to measure efflux. One of the obvious disadvantages of this closed system is a re-circulation of Rh-123, as the amount of effluxed dye is subjected to re-uptake by the cells, resulting in the identification of apparent subpopulations of cells with fast and slow uptake and excretion. In mice, differences in Rh-123 staining have been detected in T cell subsets and attributed to variations in the activity of P-gp in aging mice: all T cells initially took up equivalent amounts of Rh-123, while efflux rates differed [53]. The subpopulation of splenocytes cells that

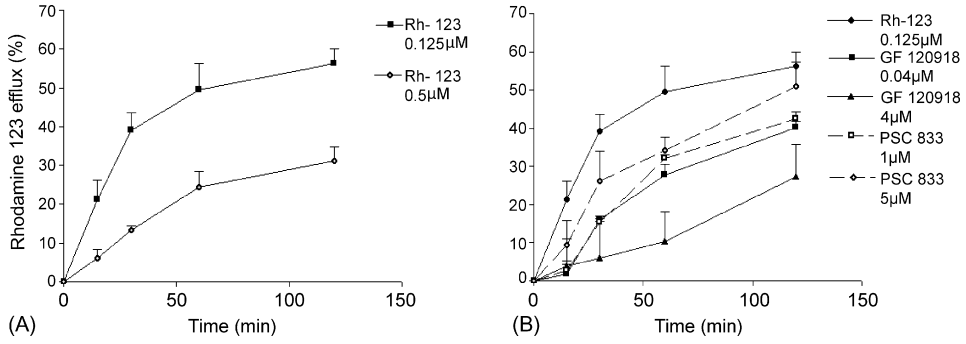

Fig. 2 – Time-dependent efflux of Rhodamine 123 (Rh-123) in chicken splenocytes. Efflux was measured over 2 h of incubation at 41 °C. (A) Concentration-dependent efflux of Rh-123 in control samples. (B) Rh-123 efflux in the presence of prototypic inhibitors. Data are presented as mean ± S.D. from six experiments.

Table 3 – Inhibition of Rhodamine 123 (Rh-123) efflux from splenocytes (subpopulation M1) as affected by inhibitors and individual fluoroquinolones (mean ± S.D. of six experiments)

Concentration of Rh-123 (μM)	Concentration of tested compounds (μM)	Inhibition of Rh-123 efflux (%)				
		Cyclosporin A	PSC833	GF120918	MK571	Ko-134
0.125	0.04	–	–	36.78 ± 1.81*	–	11.90 ± 3.53
	1	–	30.68 ± 1.22*	–	2.56 ± 0.42	12.74 ± 0.73*
	4	19.08 ± 1.28	–	70.66 ± 14.72*	–	–
	5	–	26.60 ± 4.58*	–	–	–
	20	−14.49 ± 8.12	–	–	–	–
	25	–	–	–	20.94 ± 3.69	–
0.5	0.04	–	–	46.11 ± 1.93*	–	17.13 ± 3.11*
	1	–	37.46 ± 5.30*	–	18.23 ± 4.42	14.97 ± 7.58
	4	27.74 ± 3.68*	–	68.13 ± 6.49*	–	–
	5	–	26.23 ± 4.32*	–	–	–
	20	−1.25 ± 3.07	–	–	–	–
	25	–	–	–	13.61 ± 2.23	–

		Danofloxacin	Danofloxacin mesylate	Enrofloxacin base	Marbofloxacin sulfate	Ciprofloxacin
0.125	1.56	3.26 ± 3.17	No inhibition	5.60 ± 3.57	6.37 ± 6.92	6.65 ± 2.95
	3.12	4.48 ± 2.96	No inhibition	7.43 ± 4.44	10.09 ± 5.64	5.02 ± 2.64
	6.25	2.67 ± 4.57	No inhibition	6.30 ± 5.18	11.84 ± 7.98	7.26 ± 3.64
	50	10.54 ± 3.57	8.49 ± 4.22	12.10 ± 7.80	8.15 ± 4.45	2.27 ± 2.77
0.5	1.56	9.34 ± 3.56	10.77 ± 4.26	11.27 ± 6.94	7.25 ± 5.42	8.00 ± 5.30
	3.12	13.42 ± 9.51	14.40 ± 4.78*	11.42 ± 6.02	10.05 ± 5.97	13.47 ± 10.12
	6.25	15.96 ± 4.75*	10.94 ± 3.78	10.52 ± 4.85	4.11 ± 9.42	No inhibition
	50	16.43 ± 4.23*	15.99 ± 4.43*	10.11 ± 8.13	6.13 ± 3.39	4.09 ± 6.33

* Statistically significant difference in comparison to controls at $P < 0.05$.

apparently continued the uptake of Rh-123 (denoted M2) could not be characterized in detail, but seem to have a limited capacity for dye efflux. Hence, this population was excluded in the interpretation of the results.

P-gp is the most widely studied ABC-transporter in relation to drug kinetics in mammals, but its function is poorly characterized in avian tissues [15]. Therefore, it was one of the aims of the present study to characterize avian P-gp in isolated splenocytes. PCR analysis had revealed that these splenocytes expressed also MRP2 and BCRP mRNA, suggesting that the same model can be used also for functional studies involving other transporters [1,17,34,43].

The use of Rh-123 in flow cytometry as a probe for human P-gp activity is considered to be specific, although it cannot entirely be excluded that other proteins, such as MRP1 contribute to its transport as well [10,44,45,52], whereas with regard to BCRP only a mutant of human BCRP has been found to be involved in Rh-123 transport [13].

Comparing the obtained data from poultry splenocytes with findings from other animal species [25], it is remarkable that Rh-123 efflux by chicken splenocytes is readily saturable as demonstrated by the non-linearity of Rh-123 efflux at higher concentrations including those (4 μM) found to be optimal for transport studies in lymphocytes from mammalian species.

Known inhibitors of mammalian ABC-transporters were used to further characterize Rh-123 efflux. Cyclosporin A is a broad-spectrum MDR substrate and modulator of the activity of P-gp and MRP2, whereas conflicting data exist for its inhibitory effect on BCRP [16,19,20,35,47]. A significant inhibition of the Rh-123 efflux in chicken splenocytes by cyclosporin A was only observed at a concentration of 4 μM cyclosporin A, whereas higher concentration (20 μM) did not affect Rh-123 efflux, suggesting both inhibition and stimulation of efflux [28,46]. GF120918 and PSC833 are potent inhibitors of mammalian P-gp function, with a high specificity of PSC833 for P-gp, while GF120918 is also an inhibitor of human BCRP albeit with lower potency [27,32,50]. In consideration of these previous findings, it can be suggested that the inhibition of Rh-123 efflux by PSC833 and GF120918, at the lower concentration of 0.4 μM, are mainly attributable to the inhibition of P-gp. When GF120918 was applied at a concentration equal to or exceeding 4 μM, a more pronounced inhibition was observed as compared to PSC833 and the lower concentration of GF120918. The latter findings suggested an additional role for BCRP in Rh-123 efflux. Therefore, we tested also Ko-143, a highly potent inhibitor of human and rodent BCRP [11]. Ko-143 applied at the concentrations 0.04 μM and 1.0 μM decreased Rh-123 efflux to the same extent, however, at a lower level than was observed for cyclosporin A, PSC833 and GF120918. Although the measured inhibition was not dose-dependent, these data suggest an additional role for BCRP in Rh-123 efflux. This finding is remarkable, as wild-type human BCRP was not found to be involved in Rh-123 transport. MK571 inhibited Rh-123 efflux in the splenocytes from chicken as well. MK571 is a putative inhibitor for MRPs, inclusive MRP2 [29], but data on its effect on BCRP are lacking.

Taken together, these results suggest that Rh-123 efflux in chicken splenocytes is mediated predominantly by the chicken homologues of P-gp and to a lesser extent by BCRP, while the role of members of the MRP-family need further attention.

To determine the general applicability of the presented model with avian splenocytes, selected fluoroquinolone antimicrobials (including danofloxacin, danofloxacin mesylate, enrofloxacin sulphate, marbofloxacin and ciprofloxacin) were tested for their effect on Rh-123 retention. Many fluoroquinolones have previously been found to be substrates for multiple transporters in rodents and humans. A significant inhibition of Rh-123 efflux was only observed with danofloxacin and danofloxacin mesylate. This inhibitory effect occurred at concentrations that are reached in the systemic circulation after oral application of the recommended dose regime, which is higher than that of other fluoroquinolones and based on the concept of concentration-dependent effects and resistance avoidance. An even more pronounced inhibitory effect was observed at higher dosages, which might be achieved in the gastrointestinal tract as well as in the alveolar space. All other tested drugs (enrofloxacin sulphate, marbofloxacin and ciprofloxacin) only tentatively inhibited Rh-123 efflux. These findings suggest that fluoroquinolones are in general weak inhibitors for efflux transporters. Hence, the risk for undesirable drug–drug interactions seems to be low. The finding that some fluoroquinolones are inhibitors for efflux transporters, suggests that they are substrates as well that could explain their specific kinetic behaviour, including the high concentrations in the gastrointestinal tract after parenteral administration, and the high levels in the alveolar fluid.

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