PK-PD Modeling of Fluoroquinolones and ABC Transporters in Poultry

by

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PK-PD Modeling of Fluoroquinolones and ABC Transporters in Poultry

PK-PD modellen voor fluoroquinolonen en ABC transporters in pluimvee

(met een samenvatting in het Nederlands)

Proefschrift

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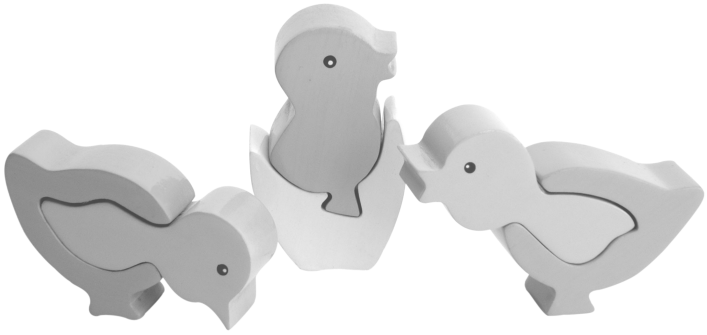
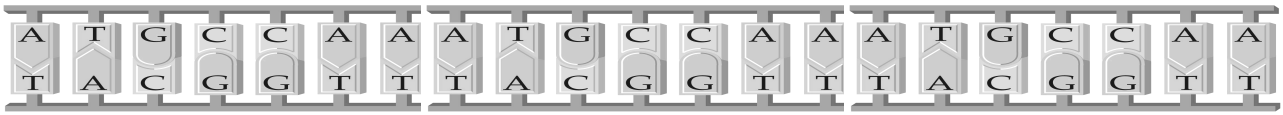
CONTENT

Abbreviations

ABC	ATP-binding cassette
ADE	Absorption, distribution and elimination
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
bw	Body weight
CFU	Colony forming units
CYP	Cytochrome P450
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
i.v.	Intravenous
IFNγ	Interferon γ
IL	Interleukin
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
mRNA	messenger RNA
MRP	Multidrug resistance protein
NBD	Nucleotide binding domain
NTCP	Sodium-dependent taurocholate cotransporter
OATP1 and OATP2	Organic anion transporting polypeptide 1 and 2
OCT	Organic cation transporter
p.o.	Oral
PBMC	Peripheral blood mononuclear cells
PK-PD modeling	Pharmacokinetic-Pharmacodynamic modeling
Rh-123	Rhodamine 123
RT-PCR	Real-time polymerase chain reaction
TM	Trans membrane
TNFα	Tumor Necrosis Factor α

CHAPTER 1





1

General Introduction

Fluoroquinolones are antibacterial drugs widely used in veterinary medicine. Due to their preferable pharmacokinetic properties, low toxicity and broad spectrum of activity against pathogenic microorganisms, various fluoroquinolones have been licensed for the treatment of animal diseases (Barbour *et al.*, 1998; Sarkozy, 2001; Webber and Piddock, 2001). Although being widely used in treatment of poultry diseases, the kinetic properties, especially in relation to the intended therapeutic use, are still incompletely investigated, and factors which may influence the optimal dosage regimens remain to be characterized.

Chemistry, pharmacokinetic and pharmacodynamic properties of fluoroquinolones in relation to their use in the therapy of poultry diseases

Chemistry and Mode of Action

Enrofloxacin, danofloxacin and marbofloxacin belong to the third generation of fluoroquinolones, developed for veterinary use only. Their chemical structure is derived from or related to the primary quinolones such as nalidixic acid (enrofloxacin, danofloxacin and marbofloxacin) and oxolinic acid (Sarkozy, 2001). The introduction of a fluorine atom at position 6 of the quinolone carboxylic acid nucleus increases the inhibitory potency against bacterial topoisomerases (gyrases) and reduced the minimum inhibitory concentration (MIC) against Gram-negative pathogens by nearly a factor of 100. At the same time, the spectrum of modern fluoroquinolones comprises also various Gram-positive pathogens (Sarkozy, 2001; Andersson and MacGowan, 2003). The chemical groups at positions 1, 5 and 7 are related to potency, spectrum and pharmacokinetic properties, whereas ligands at positions 3 and 4 determine gyrase binding affinity and uptake. The addition of piperazine ring improves the activity against Gram-negative organisms and it has been suggested that this constituent may play a role in inhibiting bacterial efflux mechanisms (Andersson and MacGowan, 2003).

Enrofloxacin, danofloxacin and marbofloxacin have amphoteric properties based on the carboxyl group at position 3 (accounting for an acid character) and the piperazine ring (accounting for a basic character). The presence of a C_2H_5-group attached to the piperazine ring of enrofloxacin enhances tissue penetration and decreases central nervous system toxicity. Danofloxacin was primarily rejected for medical use due to unfavourable kinetic data, but is licensed now in veterinary medicine as mesylate, as in this form it shows a favourable high tissue distribution. Marbofloxacin has an additional methyl group at the para-position on the piperazine ring, which increases its distribution volume and lipid solubility. Its oxadiazine ring, which is unique for marbofloxacin seems to account for the excellent bioavailability and its long elimination half-life (Fig. 1).

Quinolones inhibit the binding of topoisomerase II (gyrase) or topoisomerase IV to DNA by forming a complex with the enzymes resulting in conformational changes and loss of activity. Topoisomerases facilitate relaxation and supercoiling of DNA during transcriptional processes, and inhibition of this process results in a bactericidal effect

(Drlica and Zhao, 1997; Khodursky and Cozzarelli, 1998). The primary target of fluoroquinolones in Gram-negative species such as *E. coli* and *Neisseria gonorrhoeae* is the topoisomerase II (gyrase), whereas in Gram-positive organisms, such as *S. aureus* and *S. pneumoniae* it is topoisomerase IV. The chemical structure of the quinolone determines the preference for either target (for review see Hawkey, 2003).

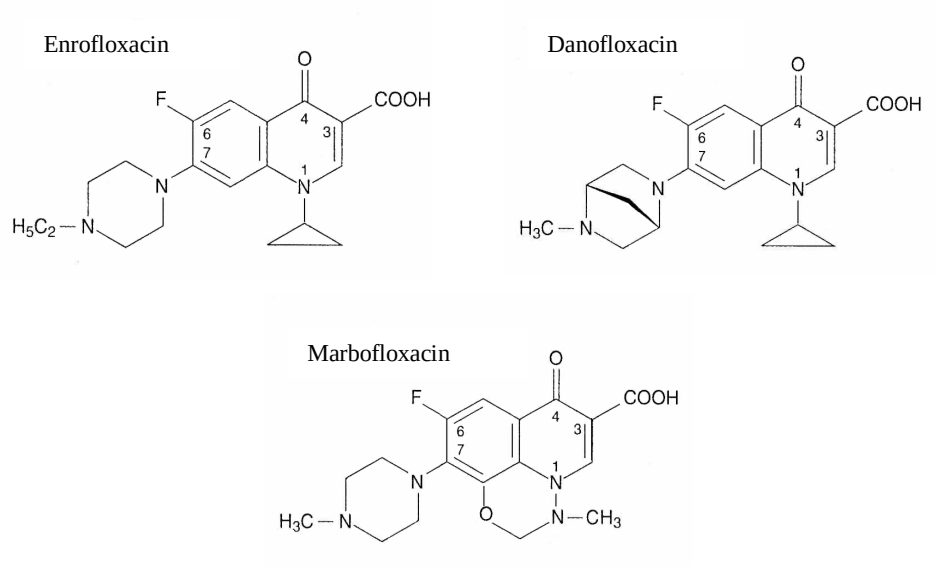


Figure 1. Chemical structure of prominent fluoroquinolones licensed for clinical use in veterinary medicine.

Spectrum of antimicrobial activity

Members of the third generation of fluoroquinolones have an excellent activity against *Enterobacteriaceae*, Gram-negative bacteria including *E. coli, Klebsiella spp., Citrobacter spp., Serratia spp., Haemophilus influenzae, B. fragilis* and *Pseudomonas aeruginosa* with MIC values between 0.03-0.12 µg/ml; good to moderate activity against *Staphylococci, Mycoplasma spp, Legionella spp, Mycobacteria, Chlamydia,* and *Ureaplasma* with MIC values varying between 0.008-0.06 µg/ml; but little or no activity against streptococci, anaerobic bacteria and variable activity against enterococci (Sarkozy, 2001; Andersson and MacGowan, 2003).

Investigations with field isolates from poultry species revealed for example that enrofloxacin and danofloxacin have similar activities (MIC_{90}, 0.05 to 1.0 µg/ml) against mycoplasma strains (Hannan *et al.*, 1997). The MIC_{90} values for danofloxacin do not exceed 0.25 and 2 µg/ml for Gram-negative (*Salmonella spp.*) and Gram-positive (*Staphylococcus aureus*) poultry isolates, respectively (Geornaras and von Holy, 2001). The *in vitro* susceptibility of *Escherichia coli* isolated from poultry for enrofloxacin was 0.015

µg/ml. For danofloxacin variable results ranging from 0.03 µg/ml to 0.25 µg/ml have been reported (Raemdonck *et al*., 1992; Charleston *et al*., 1998; Van Boven *et al*., 2003).

Antimicrobial resistance

Resistance to quinolones most commonly arises as a result of mutations in the genes encoding DNA gyrase and topoisomerase IV. Additional mechanisms of resistance to fluoroquinolones relate to changes in porins and mutational changes in regulatory genes that affect the activity of a wide range of efflux pumps (Hooper, 2000; Webber and Piddock, 2003).

Pharmacokinetics in poultry

In poultry husbandry, oral administration of drugs is commonly preferred as this allows the treatment of large flocks. Therefore, sufficient absorption of the drugs following oral administration is a prerequisite for the clinical efficacy of antibiotics. The published data about the rate of absorption of fluoroquinolones are variable and suggest that this process is not very fast. For example, the mean absorption time (MAT) for danofloxacin, enrofloxacin and marbofloxacin was calculated to 1.2, 1.4 and 4.2 h, respectively (Knoll *et al*., 1999; Anadón *et al*., 2002). A comparison of the oral bioavailability shows that in chickens marbofloxacin is absorbed to a lower extent (F=56.8%) as compared to enrofloxacin and danofloxacin (F=64-99%) (Anadón *et al*., 1995; Knoll *et al*., 1999; Haritova *et al*., 2004). The high bioavailability measured for these fluoroquinolones indicates a low first-pass effect, with the exception of enrofloxacin, which is rapidly converted into ciprofloxacin.

The relatively high volume of distribution (1.33 – 5 – 13.7 l/kg), and low rate of total body clearance (0.17 – 1.41 $l.kg^{-1}.h^{-1}$) of fluoroquinolones result in relatively long terminal half-live allowing an administration once daily (Anadón *et al*., 1997; Garcia Ovando *et al*., 1999; Knoll *et al*., 1999; Anadón *et al*., 2002). Danofloxacin and marbofloxacin, like other fluoroquinolones, are sufficiently lipid-soluble to penetrate tissues in broiler chickens. Knoll *et al*. (1999) and Anadón *et al*. (2002) found higher concentrations of these drugs in tissues than in plasma.

The metabolism of the fluoroquinolones in poultry was studied mainly by Anadón *et al*. (1995, 2002). The metabolism of danofloxacin is minimal and all postulated metabolites remained under the limit of detection when animals were treated in the normal dose range (Anadón *et al*., 1995; Knoll *et al*., 1999) Marbofloxacin is partly converted into an oxidized metabolite (N-desmethyl marbofloxacin), which has similar kinetic properties as the parent compound (Anadón *et al*., 2002). Somewhat different is the fate of enrofloxacin, which is rapidly transformed into the pharmacologically active de-ethylated product ciprofloxacin (Anadón *et al*., 1995). Fluoroquinolones are primarily excreted via the kidneys by glomerular filtration and tubular excretion by the organic anion transport system (Drusano *et al*., 1986; Bregante *et al*., 1999). Renal failure and impairment of tubular secretion usually do not cause significant changes in pharmacokinetics of fluoroquinolones due to the

existence and compensation via other routes of excretion (e.g. via biliary excretion and intestinal excretion; Wolfson and Hooper, 1991; Lefebvre *et al*., 1998).

While the pharmacokinetics of enrofloxacin, danofloxacin and marbofloxacin has been investigated to an appreciable extend in chickens as presented above, data in other avian species, as for example in turkeys, are scarce.

PK-PD modeling and optimisation of fluoroquinolone use

Fluoroquinolones have shown efficacy against a variety of bacterial diseases and one of the major indications for the use in poultry is the control of systemic colibacillosis (Hafez *et al*., 1990; Jones and Erwin, 1998; Fiorentin *et al*., 2003). Subsequently, enrofloxacin, danofloxacin and sarafloxacin have been studied in extensive field trials to demonstrate their efficacy in the treatment of colibacillosis in chickens and turkeys (Bauditz, 1987; Behr *et al.*, 1988; Ter Hune *et al*., 1991; Charleston *et al*., 1998; Chansiripornchai and Sasipreeyajan, 2002). The efficacy of danofloxacin has also been evaluated in poultry mycoplasmosis (Jordan *et al*., 1993; Liu and Fung, 1997; Barbour *et al*., 1998) and salmonellosis (Pedersen *et al.*, 2002). Evaluation of the clinical efficacy was based on clinical outcome only. However, an increased resistance to fluoroquinolones has been recognized as an important problem in poultry husbandry during last decade (EU, 2003). The increase in resistance against fluoroquinolones in poultry seems to be related to their abundant (over)use in veterinary practice, and to the administration of sub-optimal doses of these drugs. Subsequently, an increased incidence of chromosomal mutations in *gyrA, parC*, and other genes associated with efflux pumps and outer membrane permeability, have been demonstrated in field isolates obtained from poultry flocks (Ma *et al*., 1993; Everett *et al*., 1996; Mazzariol *et al*., 2000; Webber and Piddock, 2003).

In consideration of the possibility that resistance induction and development is often associated with the use of an inadequate dosing regimens, fluoroquinolones were subjected to pharmacokinetic-/pharmacodynamic (PK-PD) modeling studies. The integration of PK (bioavailability and clearance) and PD (MIC) parameters allows predicting efficacy and potency of a drug. It was originally developed for application in human medicine with the aim to optimise of dosage regimen and to improve cure rates while reducing the selection of resistant mutants (Scaglione, 2002; Mouton *et al*., 2005). Subsequently, this approach was introduced to optimise dosage regimens in veterinary medicine (Toutain *et al*., 2002; McKellar *et al*., 2004; Schneider *et al*., 2004). The interaction between pharmacokinetics and pharmacodynamics of fluoroquinolones can be assessed by measuring the area under the concentration–time curve over 24 h, and divide this by the MIC value (i.e. establishing the AUC/MIC ratio); the peak level divided by the MIC (C_{max}/MIC) and the cumulative percentage of a 24 h period that the drug concentration exceeds the MIC ($T_{>MIC}$) (Mouton *et al*., 2005). The value of >125 for AUC/MIC ratio and of 10 for C_{max}/MIC has been recommended to achieve high efficacy for fluoroquinolones, according to the concept of concentration-dependent antibacterial activity of this class of antibiotics (Schentag, 2000; Schentag *et al*., 2001; Drusano, 2004). Rapid eradication of the pathogens has been

reported with an AUC/MIC ratio of >250 (Forrest *et al*., 1997; Meinl *et al*., 2000; Wise, 2003). If the AUC/MIC values remained below 125, a stepwise increase in resistance and an incomplete eradication was observed (Schentag *et al*., 2001; Wise, 2003). It was concluded that the appearance of resistant mutants can be avoided if adequate attention is given to correct dosing regimens (Drusano, 2003). Despite of the fact that many data on PK-PD modeling of fluoroquinolones have been published for humans, laboratory and farm animals, corresponding data for poultry species are virtually absent. Moreover, the advanced analyses of kinetic data did show various inconsistencies between the expected and measured tissue levels and routes of excretion. One of the most prominent findings was the observation that the secretion of fluoroquinolones into the gut lumen (in therapeutically relevant concentrations) following parenteral administration obviously occurred not only by biliary excretion. Comparable observations were made when blood levels were compared with tissue levels in the lungs, as mucosal drug levels were recorded that exceeded those of the blood serum. These findings pointed towards the possibility that fluoroquinolones are substrates for efflux transporters.

ABC efflux transporters as determinants of tissue penetration and elimination of drugs

Drug absorption, distribution and elimination (ADE) are complex processes, which are governed by several factors. While in the past membrane permeability was discussed predominantly in relation to physicochemical properties of drugs such as lipophilicity, pKa value, molecular weight and solubility, it is now evident that transport proteins are major determinants of ADE (Van Bambeke *et al*., 2003). Of particular interest are efflux transporters (ABC transporters) that are expressed at physiological barriers (for example at the blood-brain barrier and the placenta), in the liver and in other tissues responsible for the detoxification and excretion of xenobiotics (Mealey, 2004; Hugnet *et al*., 2004). These efflux transporters influence intestinal absorption of drugs and toxins as well as their excretion from the systemic circulation into the gut lumen, and contribute to the elimination via bile and urine (for reviews see: Lin and Yamazaki, 2003a). Clinical data show that the efflux via ABC pumps conveys multi-drug resistance to chemotherapeutic agents (Klein *et al*., 1999; Honjo *et al*., 2001; Miller, 2002; Miyoshi *et al*., 2002; Dantzig *et al*., 2003), and modulates the response to treatment of many other structurally unrelated drugs (Ito *et al*., 1998, Greiner *et al*., 1999, Watkins 1999, Hoffmeyer *et al*., 2000).

At present, P-glycoprotein (P-gp), MRP and BCRP are considered as the main ABC transporter proteins which are involved in the process of drug's ADE. Fluoroquinolones are examples of P-gp substrates and inhibitors, which has been described as a class-specific behaviour of these drugs (Ito *et al*., 1997b; Murata *et al*., 1998; Sasabe *et al*., 1998; de Lange *et al*., 2000).

Structure, mechanism of function and regulation of expression of ABC transporters

ABC transporters are large membrane proteins. Their basic structure, as found in P-gp (ABCB1), consists of 12 transmembrane domains and two ATP-binding sites in a protein of about 1300 amino acids (Jones and George. 1998). In comparison to P-gp, MRP2 consists of 1545 amino acids having an additional amino-proximal membrane-spanning domain comprising approximately 200 amino acids (Tusnady *et al.*, 1997). In contrast, BCRP (ABCG2) is called half-transporter, consisting of a single hydrophobic membrane spanning domain predicted to contain 6 trans membrane (TM) helices preceded by a single nucleotide binding domain (NBD) (Leslie *et al*., 2005) (Fig. 2).

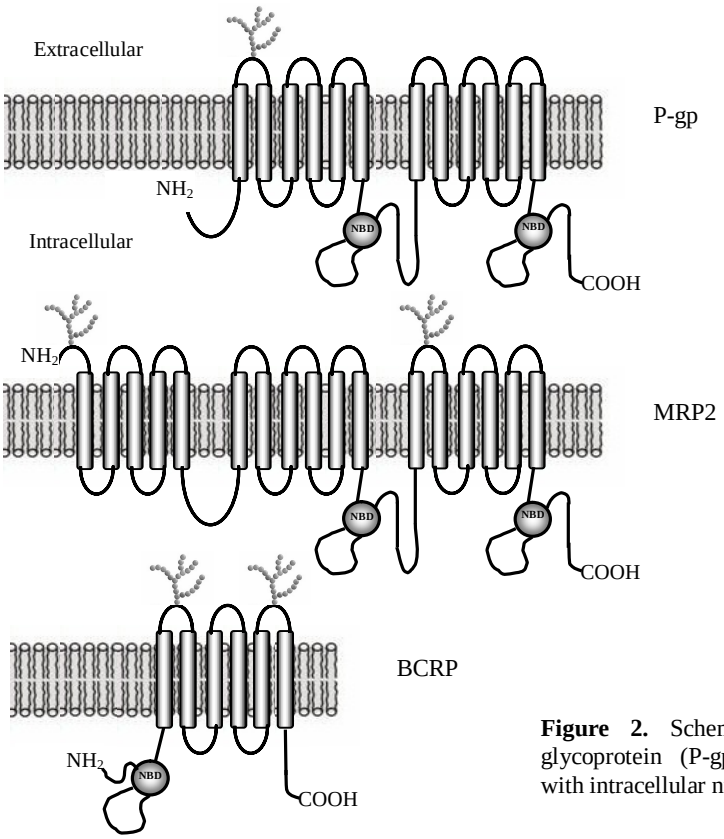


Figure 2. Schematic structure of P-glycoprotein (P-gp), MRP2 and BCRP with intracellular nucleotide binding sites.

The position and number of substrate-binding sites in ABC transporters remain to be described in more detail. It was suggested that there are at least four substrate-binding sites, which differ in substrate specificity (reviewed by Chan *et al.*, 2004). Moreover, it is suggested that the drug-binding transmenbrane regions of P-gp could form a single binding site (pocket) that binds more than one compound (Sharom *et al*., 1998; Loo and Clarke,

2005). The ability of a substrate to change the cross-linking pattern is consistent with the ability of transmembrane domains to change their shape to accommodate structurally different compounds (Schuetz *et al.*, 1996; Rosenberg *et al.*, 2001; Loo *et al.*, 2003). In the P-gp two cassettes interact cooperatively to form a single functional unit (Loo and Clarke, 1994; Muller *et al.*, 1996) (Fig. 3). The nucleotide binding domains are not required for drug binding. ATP binding and subsequent hydrolysis are essential for drug transport (Ambudkar *et al.*, 2003).

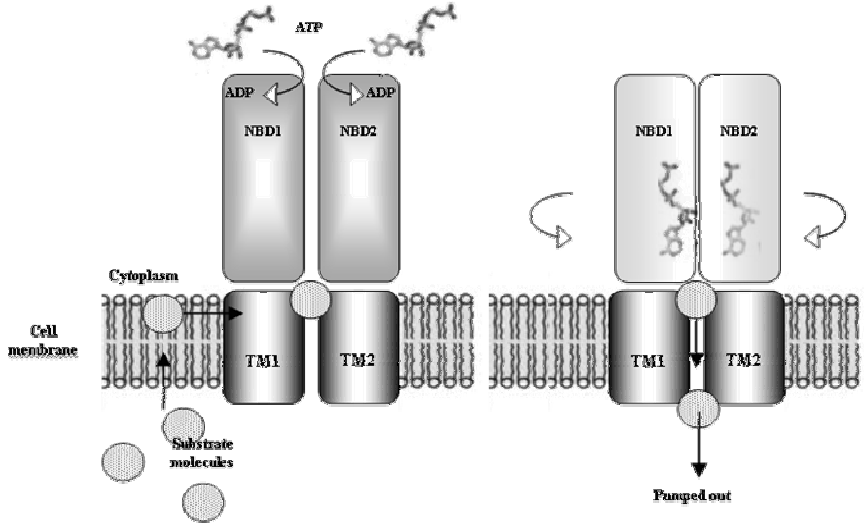


Figure 3. Schematic model of the mechanism of drug transport by P-glycoprotein.

Transcriptional regulation of many mammalian ABC transporters, including MDR1 and MRP2 is under control of so-called orphan nuclear receptors. These regulate also the expression of various biotransformation enzymes (Borst and Elferink, 2002, Di Croce *et al.*, 1999). This class of transcription factors comprises among others PXR and SXR, the pregnane- and the steroid-activated receptors, also denoted xenobiotic sensing receptors. SXR, the regulator of MDR1 expression, can be induced by various drugs such as rifampin, phenobarbital, paclitaxel, and HIV protease inhibitors (Xie *et al.*, 2000; Synold *et al.*, 2001). The antibiotic rifampin, an inducer of CYP3A4 and P-gp, increases also MRP2 mRNA and protein expression (Fromm *et al.*, 2000) via PXR and CAR (constitutive androstane receptor) (Kast *et al.*, 2002). Species differences in the level of expression seem to be associated with differences in the affinity of the receptor for various ligands (Staudinger *et al.*, 2001).

Tissue distribution of P-gp, MRP2 and BCRP

In humans and rodents, **MDR1 mRNA (ABCB1)** expression and protein levels of **P-gp** have been found in brain, kidney, testis, liver, jejunum, colon and lung, and lower levels in ileum and duodenum (Conrad *et al*., 2001; Langmann *et al*., 2003; Tang *et al*., 2004; Zimmermann *et al*., 2005). Like mammals, chickens show high levels of P-gp expression in the liver, small and large intestines, and in the kidneys (Barnes, 2001), brain, lung, heart, eye and follicles (Edelmann *et al*., 1999). Most investigations suggest that the levels of MDR1 mRNA increase progressively from the stomach to the colon, with low levels in the stomach, intermediate levels in the jejunum, and high levels in the colon (Tang *et al*., 2004; Zimmermann *et al*., 2005).

MRP2 (ABCC2) is expressed mainly at the apical membranes of epithelial cells in liver, intestines (duodenum, jejunum and ileum), and kidney tubules (Schaub *et al*., 1997; Fromm *et al*., 2000; Gotoh *et al*., 2000; Scheffer *et al*., 2002a). This protein has not been detected in colon, lung, brain and testis (Conrad *et al*., 2001). Various species differences in the level of expression of MRP2 in individual tissues have been described. For example, a high expression was found in rabbit's kidneys and intestines exceeding those of the liver (Van Aubel *et al*., 2000), whereas in mice the highest levels were detected in the intestines, followed by the liver and the kidneys (Maher *et al*., 2005).

BCRP mRNA (**ABCG2**) is detectable in many tissues: the highest levels were found in the apical membranes of placental syncytiotrophoblasts (Doyle *et al*., 1998; Jonker *et al*., 2000), the ducts and lobules of the mammary gland, venous and capillary endothelial cells of almost all tissues (Maliepaard *et al*., 2001; Scheffer *et al*., 2002b), hepatocytes (Oude Elferink, 2002), and the apical surface of enterocytes (epithelium) of the small intestines and the colon (Dietrich *et al*., 2003). Interspecies differences have been described in ABCG2 expression in the kidneys: in human kidneys, BCRP has not been detected (Maliepaard *et al*., 2001), but in rats and mice it plays an important role in the urinary excretion of sulfated compounds (Phase II conjugated metabolites) (Jonker *et al*., 2000; Mizuno *et al*., 2004).

Substrates of P-gp, MRP2 and BCRP

There is a limited number of investigations, which tried to classify compounds into **P-gp** substrates and non-substrates (Sharom, 1997; Didziapetris *et al*., 2003). For example, according to the algorithm used by Didziapetris *et al*. (2003) it can be expected that (fluoro)quinolones are substrates for P-gp because of their molecular weight (close to 400) and the fact that they are zwitterions and posses –COOH groups and ≥8 (N+O) atoms. This hypothesis was confirmed by functional studies in which ciprofloxacin, pefloxacin, grepafloxacin, levafloxacin and danofloxacin where shown to be substrates of P-gp (Seelig, 1998; Seelig and Landwojtowicz, 2000; Schrickx, 2006). Moreover, grepafloxacin, sparfloxacin, and norfloxacin have been described not only to be substrates, but also as to be strong inhibitors of the efflux of HSR-903, hence affecting the function of MRP2 (Ito *et al*., 1997a; Murata *et al*., 1998; Sasabe *et al*., 1998). Additional data show that P-gp

transports not only various structurally diverse drugs, but also endogenous steroids (Gruol *et al*., 1999; Johnstone *et al.*, 2000) and cytokines (Drach *et al*., 1996; Raghu *et al*., 1996; Veau *et al*., 2002).

Studies with hepatocytes have shown that **MRP2** is particularly involved in the excretion of a range of both, conjugated and unconjugated anionic compounds, into the bile. Among the conjugates are common Phase II metabolites such as glutathione conjugates, sulphate conjugates, and glucuronides, including bilirubin glucuronide, as well as glucuronides of fluoroquinolones, such as geprafloxacin (Sasabe *et al*., 1998; Naruhashi *et al.*, 2002). MRP2 plays thus an important role in elimination of xenobiotics (Jedlitschky *et al*., 1997; Keppler *et al*., 1998; Russel *et al*., 2002; Takikawa, 2002). Strong inhibitors of MRP2 are the leukotrien LTC4, phenolphthalein glucuronide and the compounds MK 571 and fluorescein methotrexate, which are used as prototypic inhibitors. Cyclosporine is a less potent inhibitor (Kawabe *et al*., 1999; Renes *et al*., 2000).

BCRP is a high capacity efflux transporter with wide substrate specificity, recognizing molecules of either negative or positive charge. It can extrude glucuronides and sulphate conjugates formed in enterocytes into the intestinal lumen (Adachi *et al*., 2005), but the most prominent physiological role of BCRP seem to be the mediation of excretion of drugs and xenobiotics into milk, as well as its protective role at the placental barrier.

Finally it is noteworthy to mention the overlapping substrate specificities of BCRP, MRP2 and P-gp suggesting that not only the binding affinity, but also the levels of expression in individual tissues account for the overall impact these transporters will have on absorption, distribution and excretion of endo- and xenobiotics (Taipalensuu *et al*., 2001).

Role of P-gp, MRP2 and BCRP in the pharmacokinetics of the drugs

Absorption

Taking into account the current knowledge about the level of expression of ABC transporters in different tissues it is evident that P-gp, MRPs and BCRP are important determinants of the rate of absorption of nutritional compounds as well as orally administered drugs (Hunter and Hirst, 1997; Lin *et al*., 1999a,b; Lin and Yamazaki, 2003b). Their function needs to be added to the commonly assessed factors such as solubility of drugs at different pH, diffusion and the resulting concentration gradient between intestinal lumen and mucosal blood circulation, transit time and intestinal metabolism. The expression of the individual efflux transporters along the gastro-intestinal tract seems to be variable and species-dependent, and requires more investigations. Moreover, the function of various transporters has proven to be readily saturated, already at normal therapeutic concentrations of orally applied drugs. For example, indinavir, ritonavir, and quinidine, which are excellent P-gp substrates following *in vitro* studies, show a reasonably good oral bioavailability between 60 and 80% (Polli *et al*., 2001; Raffanti and Haas, 2001), suggesting saturation of efflux transport. In *in vivo* experiments, it might be difficult to

discriminate between the rate of absorption from the gut lumen and secretion from the circulation into the gut lumen. This is exemplified by studies with digoxin: despite of the fact that digoxin is a substrate for P-gp, it has apparently a good oral bioavailability reaching 60–80% of the given dose (Ooi and Colucci, 2001). Mayer *et al.* (1996) found that orally administered and intravenously injected digoxin is excreted directly into the gut in wild type mice (16% of administered dose in 90 minutes), but hardly at all (2%) in the MDR1 (-/-) mice. Hence, further detailed investigations are required to elucidate the real impact of the level of expression and function of the transport proteins on the absorption and oral bioavailability of individual drugs.

Distribution

P-glycoprotein has a key function in the penetration of drugs through the blood-brain barrier (BBB) and in the removal of substances from the brain into the cerebral blood circulation. Therefore, it plays a decisive effect on the clinical usage of a drugs (Lin and Yamazaki, 2003a; Fromm, 2004). A classical example demonstrating this pivotal role is loperamide, an opioid widely used in the treatment of diarrhoea. It can be safely administered as it is a strong P-gp substrate and hence P-gp prevents its entrance into the brain. In turn, typical undesirable side effects of loperamide are observable in the MDR1a (-/-) mice, at common dose levels that showed no side effects in the MDR1a (+/ +) mice (Sadeque *et al.* 2000). Loperamide neurotoxicity at a common dose regimen was also reported in collies, a breed of dogs, in which mutations of the MDR1 gene, accompanied with functional losses of P-gp, is common (Sartor *et al.*, 2004).

In a model with porcine brain capillaries, which expressed P-gp and MRP2, it could be shown that both transporters contribute substantially to the active barrier function (Fricker *et al.*, 2002). With this model, the inhibitory activity of ivermectin on P-gp function could be demonstrated (Rose *et al.*, 1998). The important role of P-gp as essential factor in the function of the BBB was also recognized when ivermectin was found to reach the central nervous system in MDR1 (-/-) mice. As ivermectin is a neurotoxic compound (interacting with GABA-receptors) an unlimited diffusion into the central nervous system results in severe depression and may be fatal (Schinkel *et al.*, 1994). It is worthwhile to mention that for other compounds, such as anti-epileptics and drugs for the treatment of psychiatric disorders, the ability to pass the BBB and to reach appropriate concentrations in certain brain compartments, is a prerequisite for their therapeutic use (Löscher and Potschka, 2005)

Fluoroquinolones may induce seizures, if their penetration into the brain is not controlled by efflux pumps (Ooie *et al.*, 1997). This has been confirmed for sparfloxacin, which does enter the brain only at a very low percentage, mainly because of P-gp activity at the BBB (de Lange *et al.*, 2000). Similarly, various antibiotics cannot be used in the treatment of bacterial meningitis (e.g. cefalothin) because they are subjected to active efflux (Suzuki *et al.*, 1997).

Of therapeutic interest is also the distribution of fluoroquinolones and other antibiotics between the extracellular and intracellular space. It has been proposed that the intracellular concentration (and hence the activity) of ciprofloxacin and azithromycin could be improved by inhibiting their efflux out of macrophages. Inhibition of efflux pumps may be a useful strategy to improve the antibiotic efficacy of a wide range of antibiotics against intracellular bacteria (Seral *et al.*, 2003). Among the compounds for which drug distribution is regulated by P-gp are grepafloxacin and HSR-903, a new fluoroquinolone compound (Tamai and Tsuji, 2000).

Metabolism

Efflux transporters play a dual role in drug metabolism. Accumulating evidence indicates that Phase I biotransformation enzymes and efflux transporters are regulated in their expression by the same class of transcription factors, as mentioned above. The most prominent example is Cytochrome P450 3A4, the major cytochrome in human liver and intestines. This enzyme is likely to play a synergistic role with P-gp in suppressing the systemic bioavailability of many orally ingested compounds (Guengerich, 1995; Benet *et al*., 2004).

Moreover, Phase II conjugation products (glucuronides, glutathione conjugates, sulphate conjugates) have limited membrane permeability and hence efflux transporters have to facilitate their transport into the bile ducts, as well as central veins. The prominent role of BCRP and MRP2 in the transport of conjugated metabolites (mentioned above) is a matter of increasing interest (Sasabe *et al*., 1998; reviewed by Köning *et al*., 1999; Adachi *et al*., 2005).

Excretion

The liver and kidney play an important role in the excretion of unchanged drugs and their metabolites. The early finding that the biliary and renal clearance of digoxin is greater in MDR1a (+/+) mice than in MDR1a (-/-) mice was the first example to demonstrate the important role efflux transporters play in drug excretion (Kawahara *et al*., 1999). Studies with MDR1 (-/-) double knockout mice have demonstrated also that P-gp significantly contributes to the biliary secretion of both doxorubicin and vinblastine (Van Asperen *et al.* 2000). In turn, the P-gp inhibitors erythromycin and ketoconazole reduced the biliary clearance of, for example, fexofenadine (Ayrton and Morgan, 2001).

As discussed already above in the chapter on drug absorption, efflux transporters facilitate also the excretion of drug from the systemic circulation into the gut lumen. This was first experimentally demonstrated in mice after i.v administration of paclitaxel, which appeared in the intestinal lumen also of animals in which the bile flow towards the intestinal lumen was interrupted by gallbladder cannulation (Sparreboom *et al*., 1997). Similar mechanisms of intestinal secretion were described for talinolol after intravenous infusion (Gramatte and Oertel, 1999) and for digoxin of which approximately 10% of the

intravenous dose (1 mg) was effluxed into small intestine in healthy human volunteers (Drescher *et al*., 2003).

Another prominent example is ivermectin, which is also actively secreted from blood into the intestinal lumen. This is of clinical significance, since many gastrointestinal parasites do not feed on plasma and hence could otherwise not be reached by this anthelmintic following its parenteral application (Laffont, 2002). It could be shown that all segments of the rat small intestine excreted the parent drug after systemic administration, and the clearance of ivermectin via secretion into the small intestines was approximately 5-fold higher than the biliary clearance (Laffont, 2002).

The role of drug transporters in the modulation of antibiotic pharmacokinetics has been mainly studied for β-lactams, fluoroquinolones and, to a lesser extent, macrolides and aminoglycosides (Van Bambeke *et al*., 2003). P-gp and MRP2 for example, were shown to mediate the blood-to-lumen secretion of the fluoroquinolone grepafloxacin in the rat intestines (Naruhashi *et al*., 2001; 2002; 2003), as well as its transport by human intestinal Caco-2 cell monolayers (Yamaguchi *et al*., 2000; Lowes and Simmons, 2002). Both, MRP2 and P-gp also contribute to grepafloxacin excretion by the liver, kidney or CNS (Cormet-Boyaka *et al*., 1998; Takano *et al*., 1998; Wakasugi *et al*., 1998; Murata *et al*., 1999). In wild type Wistar rats, the biliary clearance of grepafloxacin was markedly reduced by the P-gp-inhibitor cyclosporin A (Yamaguchi *et al*., 2002). According to other investigations, the decrease in biliary and overall intestinal clearances of ciprofloxacin observed in the presence of azlocillin, cephalexin and cyclosporine can be explained by competition at the level of organic anion and/or cation transport systems, including MRP2 (Barrieáre *et al*., 1990; Dautrey *et al*., 1999).

P-gp, MRP1 and MRP2 may contribute, at least in part, to renal tubular secretion (Ito *et al*., 1997b; Sasabe *et al*., 2004). Investigations with pazufloxacin suggest that its excretion into the urine involves more active drug transporters than P-gp and MRP2 (Shimizua *et al*., 2004). Administration of aminoglycosides leads to reduction in MRP2- and P-gp-mediated efflux in renal proximal tubules, which could be an important factor in the clinical nephrotoxicity that is associated with use of this class of antibiotics (Terlouw *et al*., 2001; Miller, 2002). In addition, interactions or competition at the site of drug transporters may also account for drug-drug interactions.

Inflammatory diseases and ABC transporters

Investigation on ABC transporters during diseased conditions started with the recognition of their role in multidrug resistance in tumour cells. Increased levels of expression and changed function of ABC transporter proteins in tumour cells play an important role in anticancer therapy via the development of resistance against anticancer drugs (Leonard *et al*., 2003). Felix and Barrand (2002) found that oxidative stress, by changing P-gp expression, might also affect the transport of P-gp substrates in and out of the brain.

Endotoxin of Gram-negative microorganisms has been shown to down-regulate simultaneously hepatic P-glycoprotein, MRP2 and CYP3A and to impair the transport and biotransformation of several of their substrates (Zhao *et al*., 2002). Decreased MDR1 expression was found in liver under inflammatory conditions (Hartmann *et al*., 2001). Endotoxin-induced inflammation (using LPS) caused also a reduction in the intestinal expression and activity of P-gp, MRP2, and CYP3A in rats, which elicits corresponding changes in the intestinal transport (reduction of the basolateral to apical efflux of digoxin, amiodarone and 7-benzyloxyquinoline and a significant increase in the apical to basolateral absorption of these compounds) and metabolism of their substrates (decreased by approximately 50 to 70%) (Kalitsky-Szirtes *et al*., 2004). Inflammation provoked by infection with *Staphylococcus spp*., *E. coli*, or by LPS or cytokines (IL-1b, IL-2, IL-6, TNFα and IFNγ) decreased the level of expression of MDR1 mRNA (Fernandez *et al*., 2004). Moreover, IL10 deficient enterocolitis mice exhibit an impaired function and expression of P-gp in the epithelial cells along the intestines (Buyse *et al*., 2005). Taken together, these findings suggest changes in the pattern and level of expression in ABC transporters during inflammation, which could result in changes in drug kinetics and hence in the outcome of drug therapy (Yamada *et al*., 1996; Kawaguchi *et al*., 2000).

In conclusion, inter-species and inter-individual variations in expression of ABC efflux transporters seem to contribute to the variability in the pharmacokinetics of drugs and hence influence the therapeutic results (Lin and Yamazaki, 2003b; Benet *et al*., 2004; Sun *et al*., 2004). At present, numerous investigations are available that address the expression, functionality, and individual activity of these efflux transporters in laboratory animal species as well as humans. However, with exception of the prominent example of inherited P-gp mutations in certain dog breeds, the corresponding knowledge on drug efflux transporters is very limited in veterinary target. In turn, only very few studies have examined the levels of P-gp expression in poultry and their influence on the pharmacokinetics and pharmacodynamics of drugs (Edelmann *et al*., 1999; Barnes, 2001).

Scope of the thesis

The usage of fluoroquinolones has become common in human and veterinary medicine because of their broad spectrum and appreciable kinetic properties. In contrast to the numerous investigations in laboratory animals, humans and certain farm animal species, the available information on the pharmacokinetics in poultry species is limited. In particular, PK-PD studies, integrating pharmacokinetic and pharmacodynamic data are virtually lacking in poultry species. However, in the light of the increasing problem of antibacterial resistance, which is usually related to erroneous applications or overuse of certain fluoroquinolones (EU, 2003; Giraud *et al*., 2001; Johnson *et al*., 2002, 2003; Webber and Piddock, 2003) more data supporting the selection of an optimal dose regimen for individual fluoroquinolones are needed. Hence, we applied the principles of PK-PD modeling in the design of dosage regimen of fluoroquinolones in poultry, using two

different fluoroquinolones, danofloxacin and marbofloxacin. Subsequently we here describe the

- Pharmacokinetic-pharmacodynamic (PK-PD) modeling of danofloxacin in turkeys **(Chapter 2)**
- Integration of pharmacokinetic and pharmacodynamic indices for marbofloxacin in turkeys **(Chapter 3),** as well as the
- Implantation of tissue chambers in turkeys: A pilot study **(Chapter 4)**

More recently, fluoroquinolones have been identified as substrates for various efflux transporters (ABC transporters) in rodents as well as in humans, and these efflux transporters are known to modulate absorption, and tissue distribution of many drugs. Again, the knowledge about drug transporters is limited in avian species. The following chapters describe basic (levels of expression in healthy untreated animals) as well as applied (modulation by therapeutic application of fluoroquinolones in healthy and diseased animals) studies, addressing the relevance of efflux transporters. In detail, we describe here the

- Expression of MDR1 (P-gp) and MRP2 in tissues of healthy chickens **(Chapter 5)**
- Evaluation of P-gp, MRP2 and BCRP activity in chickens using splenocytes in an *ex vivo* model (**Chapter 6**)
- Effects of the fluoroquinolone danofloxacin on the expression of MDR1 (P-gp), MRP2 and BCRP in tissues of healthy turkeys **(Chapter 7)**, and the
- Impact of the application of the fluoroquinolones danofloxacin and enrofloxacin on the level of expression of MDR1 (P-gp) and MRP2 in chickens, infected with *E. coli* O78:K80 **(Chapter 8)**

The experimental chapter are preceded by a **General Introduction (Chapter 1)** and the overall results are put into perspective in a **General Discussion (Chapter 9).**

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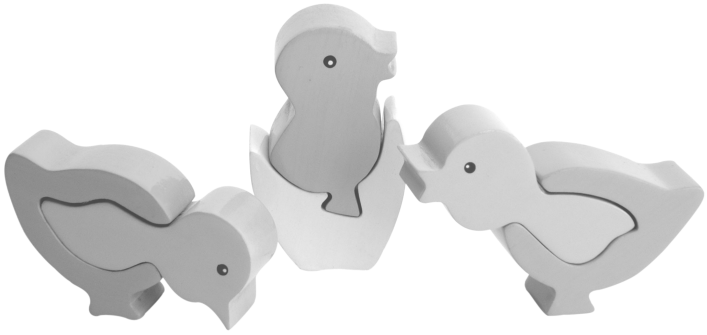
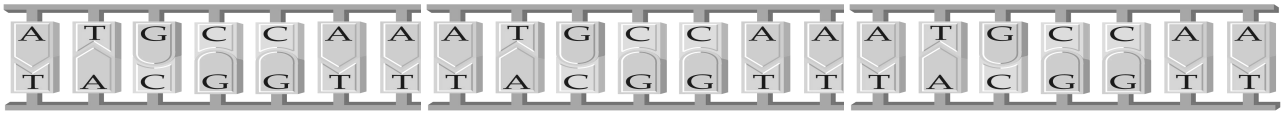
CHAPTER 2





2

Pharmacokinetic-Pharmacodynamic Modeling of Danofloxacin in Turkeys

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Abstract

Colibacillosis is a systemic disease responsible for important economic losses in poultry breeding and fluoroquinolones, including danofloxacin, are used to treat the diseased animals. The purpose of the present study was to estimate pharmacokinetic-pharmacodynamic (PK-PD) surrogates for bacteriostasis, bactericidal activity and bacterial elimination against *Escherichia coli* O78/K80, using a PK-PD approach, for danofloxacin in turkeys after oral administration. Eight healthy turkeys, breed BUT 9, were included in a two way cross-over study. The drug was administered intravenously (i.v.) and orally at a dose rate of 6 mg/kg bw. The values of the elimination half-life and the total body clearance after i.v. administration were 8.64±2.35 h and 586.76±136.67 $ml.h^{-1}.kg^{-1}$, respectively.

After oral administration the values of the absolute bioavailability and the elimination half-life were 78.37±17.35 % and 9.74±2.93 h, respectively. The minimum inhibitory concentration against the investigated strain in turkey serum was 0.25 µg/ml, four times higher than in broth. The lowest effective *ex vivo* AUC_{24}/MIC ratio required for bacteriostasis, bactericidal activity, and total killing of *E. coli* O78/K80 were 0.416 h, 1.9 h and 6.73 h, respectively. The oral dose of 6 mg/kg, used in the present study, could be interpreted as being sufficient to eliminate *E. coli* with a MIC≤0.25 µg/ml. However, considering the demand that antimicrobial resistance should be avoided by complete biological elimination, PK-PD considerations suggest that even a higher dose of 32 mg/kg/day or 0.7 mg/kcal per day should be evaluated in clinical trials.

Introduction

Danofloxacin belongs to the group of synthetic fluoroquinolone compounds, developed for veterinary use (Garcia *et al.*, 2000). As a fluoroquinolone, it acts predominating by inhibiting the enzyme topoisomerase II hence suppressing DNA and RNA replication. Fluoroquinolones result in concentration-dependent killing of many Gram-negative microorganisms (Wolfson and Hooper, 1985; Raemdonck *et al.*, 1992; Knoll *et al.*, 1999; Aliabadi and Lees, 2001; Sarasola *et al.*, 2002).

Colibacillosis is a systemic infection responsible for important economic losses in poultry breeding and fluoroquinolones are used to treat this disease (Webber & Piddock, 2001; Chansiripornchai and Sasipreeyajan, 2002; Fiorentin *et al.*, 2003). The efficacy of fluoroquinolones against *E. coli* infections administered via drinking water has been reported for enrofloxacin in chickens (Bauditz, 1987) and turkeys (Behr *et al.*, 1988; Hafez *et al.*, 1990), for danofloxacin in chickens (Ter Hune *et al.*, 1991), and for sarafloxacin in chickens and turkeys (Chansiripornchai and Sasipreeyajan, 2002). Under field conditions these fluoroquinolones can be applied with the drinking water in poultry in a flexible manner without compromising efficacy (Charleston *et al.*, 1998).

Increasing concerns have been expressed about the abundant use of fluoroquinolones in poultry, as resistant zoonotic bacteria, like *Salmonella spp.* and *Campylobacter spp.* may reach the consumer (EU, 2003). A relationship has been reported between septicaemic human and animal *E. coli* strains (Giraud *et al.*, 2001; Johnson *et al.*, 2002; Russo and Johnson, 2003; Johnson *et al.*, 2003), substantiating the demand for a prudent use of these compounds (Khac *et al.*, 1996; EU, 2003).

The ability to achieve clinical efficacy and to minimize the selection and spread of resistant pathogens depends on the relationship between the pharmacokinetic and pharmacodynamic properties of antibiotics, including fluoroquinolones (Toutain *et al.*, 2002; Wise, 2003). The application of the PK-PD approach is a potential tool to enhance the level of information and to direct the decision making process for the use of fluoroquinolones (Schentag *et al.*, 2001; Meibohm and Derendorf, 2002; Toutain, 2003). PK-PD experiments with danofloxacin have been conducted in calves, goats, sheep and camels (Aliabadi and Lees, 2001; Sarasola *et al.*, 2002; Aliabadi and Lees, 2003; Aliabadi *et al.*, 2003a; Aliabadi *et al.*, 2003b). Pharmacokinetic investigations with danofloxacin have also been described in chickens (Anadón *et al.*, 1997; Knoll *et al.*, 1999; el-Gendi *et al.*, 2001), but there are no reports about PK and PK-PD trials in turkeys.

The aim of the present study was to estimate PK-PD surrogates required for bacteriostasis, bactericidal activity and total bacterial elimination using PK-PD approach for danofloxacin in turkeys after oral administration.

Materials and methods

Drugs

Danofloxacin mesylate (Advocin 180, Pfizer, NL 9945 UDA, Part. No 2058806, V0704) was used for i.v. treatment as an 18% commercially available solution. The same sterile solution was diluted with sterile water to 1.8% w/v and than used for oral administration.

Animals

Eight healthy turkeys (breed BUT 9), 8 months old were included in the experiments. Four birds were male and four were female, weighing 8.4-10.5 kg and 6.03-7.6 kg, respectively. All birds were obtained from an experimental poultry farm in Stara Zagora, Institute of Animal Husbandry.

The animals were housed under identical conditions (at 20^{o}C), according to the requirements of the species. Standard commercial feed (without antibiotics and coccidiostats) and water were supplied *ad libitum.*

Study design

A two-way cross-over design was used, with a washout period of 15 days between individual treatments. The i.v. administration was done in the *V. brachialis*, the oral administration by installation of danofloxacin solution into the crop via a plastic tube. Blood samples were collected from *V. brachialis.* After i.v. administration blood samples were collected from the contralateral vein.

Danofloxacin was administered i.v. and orally at a dose rate of 6 mg/kg bw according to the manufacturer's instructions. Blood samples (1 ml) were collected prior to each treatment and at 0.083, 0.25, 0.5, 1, 2, 3, 6, 9, 12, 24, 36 and 48 h after the i.v. administration. Blood samples after oral administration were collected prior to each treatment and at 0.25, 0.5, 0.75 (1 ml) and at 1, 1.5, 2, 3, 6, 9, 12, 24, 36 and 48 h (1.5 ml) after dosing. They were collected in tubes without an anticoagulant and kept at room temperature for 2 h. Serum was collected after centrifugation at 1800x*g* for 15 min and stored at -25^{o}C prior to analyses.

Determination of MIC and MBC values

Bacterial isolates

For the determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC), an *Escherichia coli* O78/K80 strain isolated from turkeys was obtained from National Scientific and Diagnostic Institute of Veterinary Medicine, Sofia, Bulgaria. The used *E. coli* strain was stored on beads at -70^{o}C. Prior to use, *E. coli* was grown freshly on tryptone soya blood agar (TSA; Becton Dickinson and Co, Difco Laboratories, Le Pont de Claix, France; Ref. No 236950). Colonies from overnight growth were directly suspended in Mueller-Hinton broth (MHB; Becton

Dickinson and Co, Difco Laboratories, Le Pont de Claix, France; Ref. No 275730) to obtain turbidity comparable to that of the 0.5 McFarland turbidity standard. Cultures were diluted 1:100 with broth to obtain a dilution of 10^6 colony-forming units (CFU)/ml used as stock solution for the incubations.

Comparison of MIC in broth and in serum

Danofloxacin solution at twice the required final concentration of 128 µg/ml was added either to MHB or to serum obtained from control animals. Serial dilutions from this solution were prepared in broth and in serum with concentration ranging between 64 µg/ml and 0.0156 µg/ml to determine the value of MIC and were inoculated with approximately $5x10^5$ CFU/ml *E. coli* O78/K80. Tubes were incubated at 35°C for 18 h and then shaken to mix the contents.

An aliquot of 100 µl from each non-turbid tube was subcultured onto TSA, and the plates were incubated at 35°C overnight prior to counting of the colonies. The limit of detection was 10 CFU/ml. MICs were defined as the lowest concentration at which bacterial numbers remained below the original inoculum level. MBC was defined as the concentration at which a 99.9% reduction in the bacterial counts was achieved.

Ex vivo antimicrobial activity of danofloxacin

Colonies from overnight growth of *E. coli* O78/K80 in TSA (as mentioned above) were used to inoculate 9 ml of MHB, and the colonies were allowed to grow overnight at 35°C.

Serum samples from 6 turkeys that had received danofloxacin p.o., were collected at 0, 1, 2, 3, 6, 9, 12, 24 and 36 h and to 0.5 ml of serum was added 5 µl of stationary phase of bacterial cultures to give a final concentration of approximately $3x10^7$ CFU/ml.

To determine the number of CFU, serial dilutions were prepared with sterile saline (ranging from 10^{-4} to 10^{-6}) and incubated for 3, 6, and 24 h. Thereafter an aliquot of 20 µl was plotted on TSA plates and CFUs were counted after 16 h. The limit of detection was 10 CFU/ml.

Determination of danofloxacin serum concentrations

The serum concentrations of danofloxacin were determined by using the HPLC method of analysis as described previously by Garcia *et al*. (2000) with minor modifications. Briefly, serum samples (100 µl) were diluted with 400 µl of 0.1M phosphate buffer pH 7.4 and vortexed for 0.5 min. After adding 3 ml of dichlormethane, the samples were mixed again for 1 min and centrifuged for 6 min at 1000 *x g*, at 4°C. The organic phase was collected and was evaporated in a vacuum evaporator at 40°C. The residue was dissolved in 100 µl of demineralised Milli-Q-water. A 20 µl aliquot was injected into an HPLC system comprising a Spherisorb ODS-2-250x4.6 mm 5µM column, a High Pressure Pump Model 300 and fluorescence detector (Detector Jasco, Model 821 FP) and autoinjector (Gyna 50). Excitation and emission wavelengths were set at 280 nm and 440

nm, respectively. The mobile phase consisted of acetonitrile in aqueous solution (20:80, v/v) of potassium dihydrogenophosphate (0.02 M) and tetrabutylammonium hydrogenphoshate (0.02 M) in water. The pH was adjusted to 3.0 with phosphoric acid (85%). The flow rate was 1.0 ml/min. Peak area integrations were measured by the Chromelion Computer Program (Separations, H.I. Ambacht, the Netherlands). The limit of quantification was 0.05 μg/ml.

Standard dilutions of danofloxacin mesylate were prepared in serum obtained from untreated turkeys at concentrations of 1.0, 0.75, 0.50, 0.25, 0.10, 0.05 and 0.01 μg/ml and subjected to HPLC analysis. Linearity of standard curve was confirmed by the test for lack of fit (non significant, p=0.274) and value of *r* was 0.994. The intra-assay and the inter-assay coefficients of variation (CV) were calculated to be 10.68 and 2.27, respectively.

The extent of protein binding of danofloxacin was determined with Ultrafree-MC Centrifugal Filter Unit (Cat. No UFC3 LGC NB, Qty 250/Pk; Millipore International Holding Company BV, The Netherlands) according to the manufacturer's instructions. The concentrations of the drug in the serum and in the ultrafiltrate were determined by HPLC as described above. The free drug fraction was determined according to the equation (1):

(1) $fu=Concentration_{free}/Concentration_{total}$ (Toutain and Bousquet-Melou, 2002).

Pharmacokinetic analysis

Pharmacokinetic analysis of data was performed using a two-compartmental model (WinNonlin 4.0.1., Pharsight Corporation, 800 West El Camino Real, Mountain View, CA, USA). The best fit was determined according to the Akaike's Information Criterion. The data were weighted by the $1/y^2$ scheme. A non-compartmental analysis based on statistical moments theory (Gibaldi and Perrier, 1982) was also done. The area under the serum-concentration-time curve (AUC) was calculated by the method of trapezoids with extrapolation to infinity. The absolute bioavailability was calculated using equation (2):

(2) $F_{abs}\% = (AUC_{po}/AUC_{iv}) \times 100$.

Pharmacodynamic analysis

By using *in vitro* MIC data and *in vivo* PK parameters, the surrogate markers of antimicrobial activity, C_{max}/MIC, AUC_{24}/MIC, and $T_{>MIC}$, were determined for serum after both i.v. and oral administration of danofloxacin, according to Aliabadi and Lees (2001).

The relationship between the *ex vivo* AUC at 24 h (AUC_{24})/MIC ratio and the log10 difference between the initial bacterial count (in number of CFU per millilitre) and the bacterial count after 24 h of incubation was established for serum by using the sigmoid inhibitory E_{max} model. This model is described by the equation (3):

(3) $E = E_{max}-[(E_{max}-E_0) \times C_e^N)/(EC_{50}^N + C_e^N)]$,

where E is the antibacterial effect measured as the change in the bacterial count (in log10 CFU per millilitre) in the serum sample after 24 h of incubation compared to the initial log10 CFU per millilitre; E_{max} is the log10 difference in bacterial count between 0 and 24 h

in the control sample; E_0 is the log10 difference in bacterial count in the test sample containing danofloxacin after 24 h of incubation when the limit of detection of 10 CFU/ml is reached; EC_{50} is the AUC_{24}/MIC producing 50% of the maximal antibacterial effect; Ce is the AUC_{24}/MIC in the effect compartment (the *ex vivo* site was serum); and N is the Hill coefficient, which describes the steepness of the (AUC_{24}/MIC)-effect curve. In this investigation E_{max} represents the baseline bacterial count and E_0 is the maximal effect, because the drug inhibits bacterial growth. These PD parameters were calculated by using the WinNonlin nonlinear regression program. The PD analysis was performed on the basis of the all available data from the six animals.

Four levels of the antibacterial effect of danofloxacin were quantified from the sigmoid E_{max} equation by determining AUC_{24}/MICs required for bacteriostatic action (no change in bacterial count after 24 h of incubation), a 50% reduction in the bacterial count, a bactericidal action (99.9% decrease in the bacterial count), and the bacterial elimination as the lowest AUC_{24}/MIC that produced a reduction in the bacterial count to 10 CFU/ml in serum (limit of quantification) (Aliabadi and Lees, 2001).

Statistical analyses

The pharmacokinetic parameters of danofloxacin were presented as mean ± SD. They were computed with the Statistica 6.1 computer program (Statistica for Windows, StatSoft, Inc., USA, 1984-2002). The same program was used for statistical analysis of the standard curve.

Results

Danofloxacin MICs and MBCs.

The MIC and MBC of danofloxacin were individually estimated in MHB and in undiluted serum for a strain of *E. coli* O78/K80 isolated from turkeys that served as test organism in all experiments. The MIC in MHB was 0.06 µg/ml and in serum was 0.25 µg/ml. The MBCs were 1.0 µg/ml in serum and 0.125 µg/ml in broth.

Intravenous administration of danofloxacin.

Danofloxacin concentrations in serum.

The concentrations of danofloxacin in serum are presented in Fig. 1 and the drug concentration versus time curves were best fitted to a two-compartmental model and a summary of the kinetic parameters are given in Table 1. Serum concentrations of danofloxacin were found up to 36 h after drug administration. The protein binding was 27% (fu 0.73).

PK-PD integration for danofloxacin in serum.

Intravenous administration of danofloxacin at a dose rate of 6 mg/kg bw provided an AUC_{24}/MIC of 41 h for *E. coli* strain in serum. The time for which the concentration in serum exceeded the MIC was 13.2 h, which is more than half of the dosage interval of 24 h.

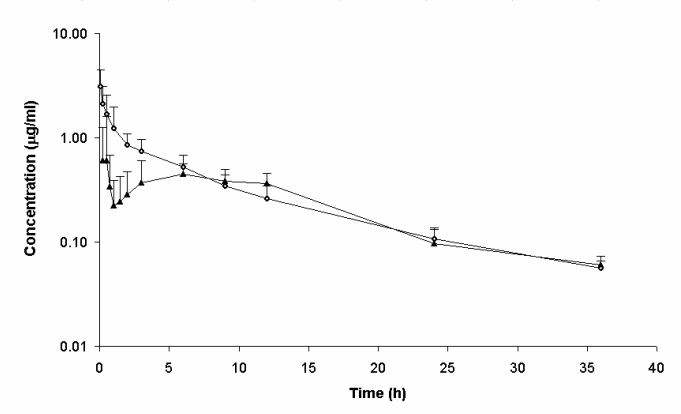

Fig. 1. Mean serum concentrations ± SD of danofloxacin mesylate (at a dose of 6 mg/kg bw, n=8) after a single i.v. (◊) and p.o. (▲) administration in turkeys.

Table 1. Pharmacokinetic parameters of danofloxacin mesylate at a dosage of 6 mg/kg bw after i.v. administration in turkeys (n=8), mean±SD.

Pharmacokinetic parameters	Units	Danofloxacin (i.v., 6 mg/kg bw)
Two-compartmental model		
α	h^{-1}	2.77 ±1.91
β	h^{-1}	0.09 ±0.03
$t_{1/2\alpha}$	h	0.36 ±0.20
$t_{1/2\beta}$	h	8.64 ±2.35
Cl_B	$ml.h^{-1}.kg^{-1}$	586.76 ±136.67
AUC	$\mu g.h.ml^{-1}$	10.77 ±2.70
MRT	h	11.01 ±3.59
Vss	$l.kg^{-1}$	6.59 ±3.15
V_2	$l.kg^{-1}$	4.45 ±2.29
V_1	$l.kg^{-1}$	2.14 ±1.07
Vd_{area}	$l.kg^{-1}$	7.43 ±3.16
Non-compartmental analysis		
MRT	h	12.16 ±3.45
AUC_{0-24h}	$\mu g.h.ml^{-1}$	10.24 ±2.37
$AUC_{0-\infty}$	$\mu g.h.ml^{-1}$	11.76 ±2.60
Cl_B	$ml.h^{-1}.kg^{-1}$	535.54 ±115.28

α - distribution rate constant; β - elimination rate constant; $t_{1/2\alpha}$ - distribution half-life; $t_{1/2\beta}$ - terminal elimination half-life; $AUC_{0-\infty}$ - area under the serum concentration-time curves from 0 h to ∞; AUC_{0-24h} - area under the serum concentration-time curves from 0 h to 24 h; Vd_{area}, Vss - area volume of distribution, steady-state volume of distribution respectively; V_1 - volume of distribution of central compartment; V_2 - volume of distribution of peripheral compartment; MRT - mean residence time, Cl_B - total body clearance.

Oral administration of danofloxacin.

Danofloxacin concentrations in serum.

The mean±SD serum danofloxacin concentrations after oral administration at a dose of 6 mg/kg bw are presented in Fig. 1. Data for serum were analysed by non-compartmental analysis (Table 2). Two peaks were found: the first peak was measured between 0.25 and 0.5 h after drug administration and the second peak was found 6 h after treatment. In some animals, maximum concentrations were measured up to 0.75 h after treatment and a second, less prominent peak was detected at 6 h after drug administration. In other animals only one peak occurring between 3 and 6 h after danofloxacin administration was observed. The value of MAT was 2.89±0.65 h.

Table 2. Pharmacokinetic parameters of danofloxacin mesylate at a dosage of 6 mg/kg bw after oral (p.o.) administration in turkeys (n=8), mean±SD.

Pharmacokinetic parameters	Units	Danofloxacin (p.o., 6 mg/kg bw)
Non-compartmental analysis		
β	h^{-1}	0.08 ±0.03
$t_{1/2\beta}$	h	9.74 ±2.93
MRT	h	15.17 ±4.37
C_{max}	$\mu g.ml^{-1}$	1.17 ±0.95
T_{max}	h	2.13 ±2.56
AUC_{0-24h}	$\mu g.h.ml^{-1}$	7.70 ±1.75
$AUC_{0-\infty}$	$\mu g.h.ml^{-1}$	8.95 ±2.0
F_{abs}	%	78.37 ±17.35

β - elimination rate constant; $t_{1/2\beta}$ - terminal elimination half-life; C_{max} - maximum serum levels; t_{max} - time of C_{max}; $AUC_{0-\infty}$ - area under the serum concentration-time curves from 0 h to ∞; AUC_{0-24h} - area under the serum concentration-time curves from 0 h to 24 h; MRT - mean residence time; F_{abs}% -absolute bioavailability.

PK-PD integration for danofloxacin in serum after oral administration.

The PK-PD integration for the *in vivo* PK data and the MIC measured *in vitro* are presented in Table 3. The value of AUC_{24}/MIC and for the ratio C_{max}/MIC for the *E. coli* strain used were 29.65±7.69 h and 4.06±4.21, respectively, when MIC in serum was set to 0.25 μg/ml. The AUC_{24}/MIC, calculated by using the MIC value of 0.06 μg/ml as determined in broth, was 123.53±32.02 h and C_{max}/MIC was 16.9±17.5. These data show that the values of AUC_{24}/MIC and C_{max}/MIC, calculated with the MIC determined in serum were nearly 4 times lower than the values of the same surrogates calculated with MIC, determined in broth. The value of T>MIC was 14.3 h.

Ex vivo antibacterial activity of danofloxacin in serum after oral administration.

The *ex vivo* activity of danofloxacin against the *E. coli* strain used in serum of treated animals was determined at seven time points using control sample and samples collected between 2 and 24 h after administration. These time points were selected

according to the danofloxacin concentrations found in serum. The bacterial time-killing curves are presented in Fig. 2. For the samples collected from all animals during the first 12 h, danofloxacin exerted a very good bactericidal effect after 6 h of incubation. After 24 h of incubation almost all bacteria were killed (detection limit, 10 CFU/ml) for all samples collected between 2 and 12 h. For samples obtained between 3 and 12 h, a bacteriostatic effect was detected after 3 h of incubation. No bacteriostatic or bactericidal effects were obtained for serum samples collected at 24 h in five animals and bacterial re-growth was determined after 6 h of incubation. A bactericidal effect was determined in one bird only for a sample obtained at 24 h and after 24 h incubation.

***In vivo* and *ex vivo* AUC_{24}/MIC of danofloxacin for *E. coli*.**

The *in vivo* and *ex vivo* AUC_{24}/MIC ratios for danofloxacin in serum after oral administration of the drug at a dose of 6 mg/kg bw are presented in Table 3. The *ex vivo* AUC_{24}/MIC values from 33.02 to 41.94 h for serum samples collected between 3 h and 12 h provided a good bactericidal effect, sufficient to eliminate bacteria almost completely after 24 h of incubation (Fig. 2; Table 3). Danofloxacin administration provided a mean *in vivo* AUC_{24}/MIC of 29.65 h for the serum samples. This was lower than the *ex vivo* AUC_{24}/MIC for serum samples collected between 3 h and 12 h and almost equal to the value for 2 h. The *ex vivo* AUC_{24}/MIC value for 24 h samples is low and was not sufficient to provoke a bactericidal effect in all animals, but allowed a bacteriostatic action in four animals and bactericidal activity in one animal.

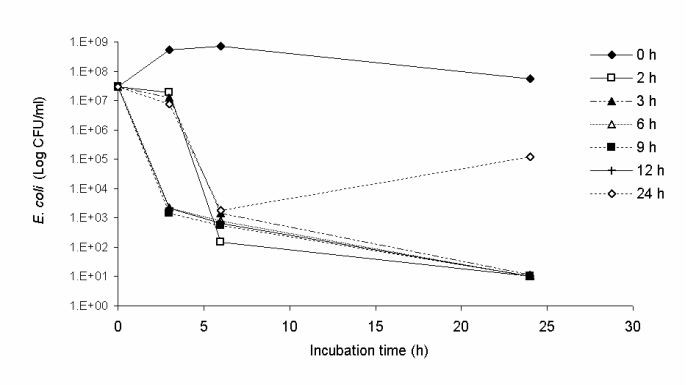

Fig. 2. *Ex vivo* antibacterial activity of danofloxacin (plots of log10 CFU per ml versus time) against E. coli in serum after p.o. administration at a dose of 6 mg/kg bw. The depicted values are means (n=6).

Table 3. *In vivo* and *ex vivo* $AUIC_{24h}$ and $AUBC_{24h}$ of danofloxacin mesylate after oral (p.o.) administration of 6 mg/kg bw in turkeys (n=6), mean±SD.

Parameter	Value (h)
Danofloxacin *in vivo*	
AUC_{24h}/MIC	29.65±7.69
AUC_{24h}/MBC	7.41±1.92
Danofloxacin *ex vivo*	
2h AUC_{24h}/MIC	24.23±17.00
3h AUC_{24h}/MIC	33.71±21.75
6h AUC_{24h}/MIC	41.94±11.97
9h AUC_{24h}/MIC	35.55±12.47
12h AUC_{24h}/MIC	33.02±10.12
24h AUC_{24h}/MIC	8.50±3.60
2h AUC_{24h}/MBC	6.06±4.25
3h AUC_{24h}/MBC	8.43±5.44
6h AUC_{24h}/MBC	10.48±2.99
9h AUC_{24h}/MBC	8.89±3.12
12h AUC_{24h}/MBC	8.26±2.53
24h AUC_{24h}/MBC	2.13±0.90

In vivo AUC_{24h}/MIC and AUC_{24h}/MBC - ratio of serum AUC_{24h} after administration of danofloxacin: MIC and MBC in serum.
Ex vivo AUC_{24h}/MIC and AUC_{24h}/MBC - ratio of serum AUC_{24h} in samples collected between 1 and 24 h from animals receiving danofloxacin and incubated for 24 h: MIC and MBC in serum.

Ex vivo AUC_{24}/MIC ratios required for bacteriostasis, bactericidal activity, and total elimination of bacteria.

The sigmoid E_{max} equation was used to estimate the values of AUC_{24}/MIC required for bacteriostasis (no change in the number of bacteria), bactericidal activity (a 3-log reduction in the bacterial count), and elimination of bacteria (a reduction in the bacterial count to <10 CFU/ml). Figure 3 presents the bacterial count and the AUC_{24}/MIC relationship for serum over 24 h. The calculated mean AUC_{24}/MICs that produced bacteriostasis, bactericidal activity, and elimination of bacteria were 0.42, 1.90, and 6.73 h, respectively (Fig. 3; Table 4). The finding that the danofloxacin AUC_{24}/MIC that produced bactericidal activity was not much higher than those that produced bacteriostasis could be explained by the steep slope of the AUC_{24}/MIC-versus-bacterial counts.

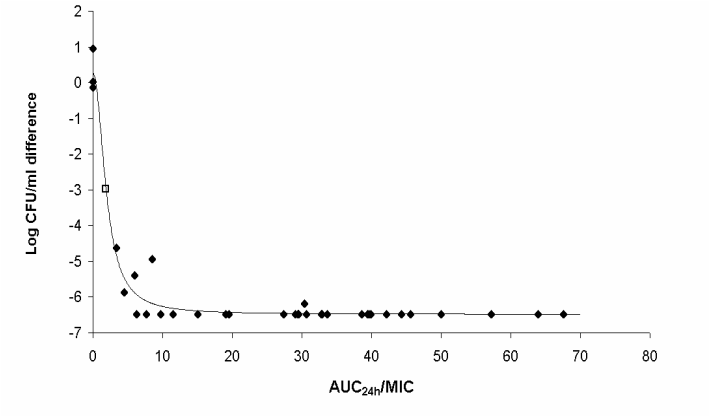

Fig. 3. Plots of *ex vivo* AUC_{24}/MIC versus bacterial count (log10 CFU per ml) for *E. coli* in serum of turkeys. The curve represents the line of predicted values, based on the sigmoid E_{max} equation and the points are the values of the individual animals.

Table 4. Integration of *ex vivo* pharmacokinetic and pharmacodynamic data obtained for danofloxacin mesylate after oral (p.o.) administration of 6 mg/kg bw in turkeys (n=6), mean±SD.

Parameter	MIC in serum 0.25 µg.ml^{-1}	MIC in broth 0.06 µg.ml^{-1}
Log E_0 (CFU.ml^{-1})	-6.48	-6.48
Log E_{max} (CFU.ml^{-1})	0.258	0.278
EC_{50}	1.96	8.35
$LogE_{max}$-$LogE_0$	6.74	6.76
Slope	2.08	2.12
$AUIC_{24h}$		
Bacteriostatic	0.416 h	1.89
Bactericidal	1.90 h	8.11
Elimination	6.73 h	27.98

Log E_0 - difference in log of number of bacteria (CFU.ml^{-1}) in sample incubated with danofloxacin between time 0 and 24 h, when the detection limit (10 CFU.ml^{-1}) is reached; Log E_{max} - difference in log of number of bacteria (CFU.ml^{-1}) in control sample (absence of danofloxacin) between time 0 and 24 h; EC_{50} ($AUC_{24\ h}$/MIC_{50}) - $AUC_{24\ h}$/MIC of drug producing 50% of the maximum antibacterial effect; *N* - the Hill coefficient; $AUIC_{24h}$ - $AUC_{24\ h}$/MIC ratio, required for bacteriopstatic, bactericidal effect and bacterial elimination.

Discussion

The therapeutic use of danofloxacin mesylate in turkeys should be based on a detailed analysis of the pharmacodynamic and pharmacokinetic properties. Danofloxacin, like other fluoroquinolones, is sufficiently lipid-soluble to penetrate tissues (Brown, 1996), which is supported by high values of Vd_{area} and V_2. Knoll *et al*. (1999) found higher concentrations of danofloxacin in tissues than in plasma of broiler chickens. The value of Cl_B in turkeys (0.535±0.12 l.kg^{-1}.h^{-1}) are in the same range as those in broiler chickens (0.26-1.41 l.kg^{-1}.h^{-1}) according to the data from Anadón *et al*. (1997) and Knoll *et al.*

(1999), and the elimination half-life was longer after i.v. administration of the drug in turkeys. After oral administration of danofloxacin in turkeys, considerably inter-individual differences in MAT and C_{max} were found. The values of C_{max}, however, were in the range of 0.47 to 1.85 µg/ml, as also published for broiler chickens (Anadón *et al.*, 1997; Knoll *et al.*, 1999). This applies also for the oral bioavailability in turkeys which was comparable to that in broilers. The double peak of the serum concentrations, found after oral administration of danofloxacin in turkeys and enrofloxacin in broilers (Sumano *et al.*, 2003), could not be conclusively explained without additional investigations, but might be related to bi-phasic crop- and/or stomach emptying.

MIC values were used to predict antimicrobial efficacy and potency and comparative *in vitro*, *ex vivo*, and *in vivo* studies, were conducted. The advantages and disadvantages of these approaches were discussed in detail by Aliabadi and Lees (2001). As a first step MICs were determined both in broth and also in undiluted turkey's serum to enable meaningful calculation of the surrogates AUC_{24}/MIC and C_{max}/MIC for danofloxacin. The difference between the MIC values obtained in turkey serum and broth was significant. Significant differences between MIC determined in broth and biological fluids have been reported previously (Aliabadi *et al.*, 2003a; Aliabadi *et al.*, 2003b), showing that the antimicrobial activity of most fluoroquinolones in the presence of serum is decreased two-fold, with the exception of trovafloxacin, for which the decrease was four-fold (Wise, 2003). The MIC value in broth found in our study was comparable with the reported MIC_{90} value for danofloxacin against *E. coli* and ranged from $\leq$ 0.015 µg/ml to 0.25-64 µg/ml (Raemdonck *et al.*, 1992; Knoll *et al.*, 1999).

Estimation of the PK-PD surrogates showed that plasma fluoroquinolone C_{max}/MIC ratios greater than 3 correlate with 99% reduction of bacterial counts, and only a C_{max}/MICs of $\geq$8 is considered to be sufficient to prevent the emergence of resistant bacteria (Drusano, 2000; Scaglione, 2002). Hence, the value of C_{max}/MIC for the investigated *E.coli* strain could be enough for a 99% reduction in bacterial counts, but it does not indicate prevention of emergence of resistant bacteria. This finding could be considered as a partial explanation for the development of resistance in some bacterial subpopulations in poultry, and for higher percentage of resistance of *E. coli* in turkeys (EMEA/CVMP/342/99-Final, 1999).

The results from clinical practice in human medicine indicate that the majority of patients with an AUC_{24}/MIC<125 were not cured and showed stepwise increases in MIC (Wise, 2003). The data from the present study show that the values of the *in vivo* AUC_{24}/MIC ratio and the lowest effective *ex vivo* AUC_{24}/MIC ratios required for bacteriostasis, bactericidal activity, and total killing of the bacteria were lower as the breakpoint value of 125. At the dose of 6 mg/kg bw, the *ex vivo* AUC_{24}/MIC value required for bacterial elimination was lower than the *in vivo* AUC_{24}/MIC ratio. The values of AUC_{24}/MIC of danofloxacin, that could ensure bacterial elimination of *M. haemolytica* and *E. coli* in mammals, were also 2 to 4 times lower than 125 h (Aliabadi and Lees, 2001; Aliabadi *et al.*, 2003a; Aliabadi *et al.*, 2003b). The slope of the *ex vivo* AUC_{24}/MIC versus bacterial count curve was steep with a relatively high Hill coefficient, which is to be expected for a drug with a concentration-dependent killing action (Toutain *et al.*, 2002). The Hill coefficient in poultry was lower than those found in other investigations with

danofloxacin in mammals (Aliabadi and Lees, 2001; Aliabadi *et al.*, 2003a; Aliabadi *et al.*, 2003b) and greater differences have to be expected between concentrations, which provoke bacteriostasis, bactericidal action and bacterial elimination. Differences in PK-PD surrogates, between turkeys and mammalian species, including humans, confirm the necessity to find the appropriate breakpoints for a bactericidal effect and bacterial elimination in birds (Drusano, 2000; Aliabadi and Lees, 2001; Lees and Aliabadi, 2002; Scaglione, 2002; Aliabadi *et al*., 2003a; Aliabadi *et al.*, 2003b).

The use of antibacterials in poultry is often associated with an incomplete bacterial eradication resulting in an insufficient clinical response in some cases, and the risk of the emerge of antibacterial resistance. Knowledge of the PK and PD properties of danofloxacin, obtained by PK-PD approach can be applied to evaluate dosing regimens. The optimal dose for danofloxacin, estimated using the equation proposed by Toutain *et al.* (2002) for treatment of *E.coli* with MIC of 0.25 μg/ml, and the lowest target AUC_{24}/MIC of 6.73 (for bacterial elimination) would be 1.73 mg/kg bw per day. The oral dose of 6 mg/kg used in this experiment was selected to eliminate *E. coli* bacteria with a MIC≤0.25 μg/ml. The results from the present study, however, suggest that even higher doses of danofloxacin should be evaluated in clinical trials. The dose that would allow the AUC_{24}/MIC ratio to exceed 125, is 32 mg/kg/day. Using the approach for dose estimation based on the metabolic rate (Dose in mg/kcal=Dose in mg/(78.Weight$^{0.75}$), Dorestein, 1999), results in a calculated dose of 0.7 mg/kcal per day.

It should however be recalled that a PK-PD approach has clear limitations. For example, the pharmacokinetics, tissue distribution and intracellular killing of bacteria by drugs can be influenced by pathophysiological changes during an infection (Van Miert, 1991); moreover the impact of factors such as host-defence mechanisms and emerge of resistance of pathogenic *E. coli* remained unconsidered (Aliabadi and Lees, 2002). Moreover, the post-antibiotic effect and the post-antibiotic sub-MIC effect phenomena in relation to the clinical efficacy and reduction of the selection of resistance could be predicted only to the certain extent by the C_{max}/MIC ratio (Drusano, 2000; Scaglione, 2002). Taking these factors into account, further experiments with either naturally diseased animals or disease models should be conducted to establish the lowest AUC_{24}/MIC that results in the elimination of bacteria *in vivo* in the diseased animal.

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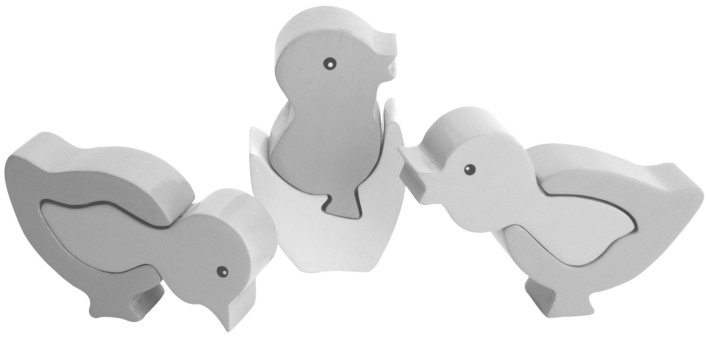
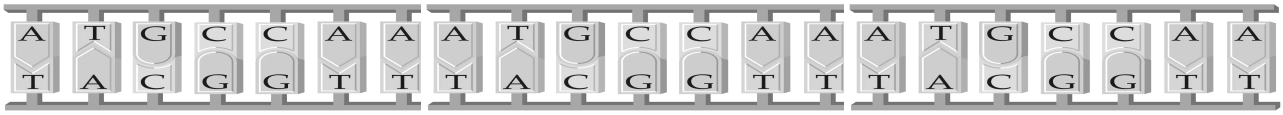
CHAPTER 3





3

Integration of Pharmacokinetic and Pharmacodynamic Indices of Marbofloxacin in Turkeys

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Abstract

Fluoroquinolones are extensively used in the treatment of systemic bacterial infections in poultry, among others for systemic *E. coli* bacillosis that is a common disease in turkey flocks. Marbofloxacin has been licensed for use in various mammalian species, but as yet not for turkeys, although its kinetic properties distinguish it from other fluoroquinolones. For example, the longer half-life of marbofloxacin in many animal species has been appreciated in veterinary praxis.

It is generally accepted that for fluoroquinolones the optimal dose should be estimated on the basis of pharmacokinetic and pharmacodynamic characteristics of the drug under consideration. Knowledge of these specific data in the target animal species allows establishing an integrated PK-PD model that is of high predictive value. In the present study, the antibacterial efficacy (PD indices) against a field isolate of *Escherichia coli* O78/K80 was investigated *ex vivo* following oral and intravenous administration of marbofloxacin to turkeys (breed BUT 9, 6 animals per group) at dose of 2 mg/kg bw. At the same time, the plasma concentrations of marbofloxacin were measured at different time intervals by a standardized HPLC method, allowing the calculation of the most relevant kinetic parameters (PK parameters).

The *in vitro* MIC of marbofloxacin against the selected *E. coli* O78/K80 strain was 0.5 µg/ml in blood serum of turkeys, and the value of C_{max}/MIC ratio was 1.34. The lowest *ex vivo* AUC/MIC required for bacterial elimination was lower than AUC/MIC. These first results suggested that the recommended dose of 2 mg/kg bw marbofloxacin is sufficient to achieve a therapeutic effect in diseased animals. However, considering the risk of resistance induction, the applied dose should be equal to AUC/MICs>125, the generally recommended dose for all fluoroquinolones. According the presented PK-PD results a dose 3.0-12.0 mg/kg bw per day would be needed to meet this criterion. In conclusion, the results of the present study provide the rationale for an optimal dose regimen for marbofloxacin in turkeys and hence should form the basis for dose selection in forthcoming clinical trials.

Introduction

Marbofloxacin is a synthetic fluoroquinolone, developed for veterinary use only (Schneider *et al*., 1996). Belonging to the third generation of quinolones it has a broad spectrum of activity (Wolfson and Hooper, 1985), and a bactericidal concentration-dependent killing is observed against many Gram-negative bacteria (Spreng *et al*., 1995; Toutain, 2002; Biksi *et al*., 2003; Thomas *et al*., 2003). The pharmacokinetic properties of marbofloxacin have been studied in several mammalian species, and some advantages over other fluoroquinolones, such as a longer elimination half-life were described (Schneider *et al*., 1996; Aliabadi and Lees, 2002; Regnier *et al*., 2003; Schneider *et al*., 2004). In practice, this would enable a single treatment per 24 h, with serum concentrations remaining above MIC for more than 12 hours. Comparable kinetic data are lacking, however, for turkeys as yet.

Fluoroquinolones are used in poultry predominantly with the aim to control systemic colibacillosis (Jones and Erwin, 1998; Webber and Piddock, 2001; Chansiripornchai and Sasipreeyajan, 2002; Fiorentin *et al*., 2003). The efficacy of this class of drugs against colibacillosis has been tested under field conditions, but results are based solely on monitoring of the clinical outcome (Bauditz, 1987; Behr *et al*., 1988; Hafez *et al*., 1990; Ter Hune *et al*., 1991; Charleston *et al*., 1998; Chansiripornchai and Sasipreeyajan, 2002). The weak point of this approach is that in field trials spontaneous clinical recovery often masks differences in bacteriological efficacy of antibacterial drugs (Pollyanna effect), resulting in the use of sub-optimal dose regimens, and hence increasing the risk of resistance induction. Particularly in poultry, sub-optimal antibacterial therapy comprises a risk for human health, as resistant zoonotic bacteria, like *Salmonella spp., Campylobacter spp.* and VTEC (verotoxin-producing *E. coli*) may reach the consumer (Johnson *et al*., 2002; EU, 2003; Johnson *et al*., 2003; Russo and Johnson, 2003). Thus therapeutic regimes need to be critically reviewed, correlating bacterial cure rates with the risk for selection and spread of resistant pathogens.

The clinical success of a given therapy depends on the relationship between the pharmacokinetic (PK) and pharmacodynamic (PD) properties of a drug (Meinl *et al*., 2000; Wise, 2003; Mouton and Vinks, 2005). The integration of PK (bioavailability and clearance) and PD (MIC) indices allows to predict efficacy and potency of a drug in the early phase of drug development and supports post-marketing surveillance (Toutain, 2002; Toutain *et al*., 2002). Hence, PK-PD models serve to select the optimal drug dosage and the more specific selection of an appropriate antimicrobial within the given class of antibiotics (Ball *et al*., 2002; Lees and Aliabadi, 2002; Meibohm and Derendorf, 2002; Toutain, 2003). Increasing evidence suggests that the main PK-PD surrogates for fluoroquinolones correlating with clinical cure and bacterial eradication are the AUC/MIC and C_{max}/MIC ratios (Schentag *et al*., 2001; Ball *et al*., 2002). Hence this approach determines *ex vivo* PK-PD indices, which subsequently allow a more targeted design in confirmatory *in vivo* studies (Aliabadi and Lees, 2001; 2002; 2003). PK-PD experiments with marbofloxacin were

previously conducted in calves, cows, goats and dogs (Aliabadi and Lees, 2002; Regnier *et al*., 2003; Waxman *et al*., 2003; Schneider *et al*., 2004). Moreover, pharmacokinetic data for marbofloxacin have been estimated for chickens and *Eurasian buzzards* (Garcia-Montijano *et al*., 2001; Anadòn *et al*., 2002). However, there are no reports about PK and PK-PD indices in turkeys, and the advantages or possible disadvantages of marbofloxacin in comparison to other fluoroquinolones were not evaluated yet.

Hence, the aim of the present study was to estimate the PK-PD surrogates required for bacteriostasis, bactericidal activity and bacterial elimination as described by Aliabadi *et al*. (2003b) and Toutain *et al.* (2002) for marbofloxacin in turkeys after oral administration, as these data provide a basis for the suggestion of optimal therapeutic dose regimes.

Material and Methods

Drugs

Marbofloxacine (Marbocyl 10 % injectable solution, Vetoquinol, Batch No 130300/1205 PdA1, V1205) was used for i.v. treatment. The same sterile formulation was diluted with sterile pyrogen-free water to 1% w/v and than used for oral administration.

Animals

Six clinically healthy turkeys (breed BUT 9), 8 months old were included in the experiments. Three birds were male and three were female, with a body weight of 9.9-10.12 kg and 6.08-6.96 kg, respectively. The animals were obtained from an experimental poultry farm in Stara Zagora, Institute of Animal Husbandry.

Animals were housed under identical conditions (at 20^{o}C), according to the requirements for this species. Standard commercial feed (without antibiotics and coccidiostats) and water were supplied *ad libitum.*

Study design

A two-way crossover design was used, with a washout period of 15 days between individual treatments. The i.v. administration was given in the *V. brachialis*, the oral administration by installation of the marbofloxacin solution into the crop via a plastic tube, after 12 hours food deprivation. Blood samples were collected from the *V. brachialis.* After i.v. administration, blood samples were collected from the contra lateral vein.

Marbofloxacin was administered i.v. and orally at a dose rate of 2 mg/kg bw, according to the manufacturer's instructions for other animal species. Blood samples were collected prior to each treatment and at 0.083, 0.25, 0.5, 1, 2, 3, 6, 9, 12, 24, 36 and 48 h after the i.v. administration. Blood samples after oral administration were collected prior to each treatment and at 0.25, 0.5, 0.75 (1 ml) and at 1, 1.5, 2, 3, 6, 9, 12, 24, 36 and 48 h (1.5 ml) after dosing. Samples were collected without anticoagulant and kept at room temperature for 2 h in the dark. Serum was collected after centrifugation at 1800×*g* for 15 min and stored at -25^{o}C prior to analyses.

Determination of MIC and MBC values

Bacterial isolates

The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values were determined with an *Escherichia coli* O78/K80 strain, isolated from turkeys, that was obtained from the National Scientific and Diagnostic Institute of Veterinary Medicine, Sofia, Bulgaria. The used *E. coli* strain was stored on beads at -20^{o}C prior to use. *E. coli* was grown on tryptone Soya blood agar (TSA; Becton Dickinson and Co, Difco Laboratories, Le Pont de Claix, France; Ref. No 236950). Colonies from overnight growth were directly suspended in Mueller-Hinton broth (MHB; Becton Dickinson and Co, Difco Laboratories, Le Pont de Claix, France; Ref. No 275730) to obtain a turbidity comparable to that of the 0.5 McFarland turbidity standard. Cultures were diluted 1:100 with broth to obtain a dilution of 10^6 CFU/ml.

MIC determination and activity in serum

Marbofloxacin solutions at twice the required final concentration of 128 µg/ml were added either to MHB (according to NCCLS, 2000) or to blood serum obtained from control animals. Serial dilutions from this solution were prepared in broth and in serum with concentration ranging between 64 µg/ml and 0.0156 µg/ml and were inoculated with approximately $5x10^5$ CFU/ml *E. coli* O78/K80. Tubes were incubated at 35°C for 18 h and then shaken to mix the contents.

An aliquot of 100 µl from each tube was subcultured on TSA, and the plates were incubated at 35°C overnight and the colonies counted. The limit of detection was 10 CFU/ml. MICs in broth and in serum were defined as the lowest concentration at which bacterial growth remained below the level of the original inoculum. MBCs in broth and in serum were defined as the concentration at which a 99.9% reduction in the bacterial counts was achieved.

Antimicrobial activity in the serum of animals treated with marbofloxacin

Eight to ten colonies from overnight growth of *E. coli* in TSA (as mentioned above) were used to inoculate 9 ml of MHB, and the colonies were allowed to grow overnight at 35°C.

To 0.5 ml serum from treated animals 5 µl of the stationary phase of bacterial cultures was added, to give a final concentration of approximately $3x10^7$ CFU/ml.

To determine the number of CFU, serial dilutions were prepared with sterile saline (ranging from 10^{-2} to 10^{-6}) and incubated for 3, 6, and 24 h. Thereafter an aliquot of 20 µl was plotted on TSA plates and CFUs were counted after 16 h incubations. The limit of detection was 10 CFU/ml.

Determination of marbofloxacin serum concentrations

HPLC method

The serum concentrations of marbofloxacin were determined by HPLC according to the method of analysis as described by Garcia *et al.* (1999). Standard solutions were prepared in serum obtained from untreated turkeys at concentrations of 2.5, 1.0, 0.5, 0.2, 0.1, 0.05, 0.025, 0.02 (LOQ) and 0.01 (LOD) μg marbofloxacin per ml. The value of *r* for the standard curve was 0.998 and the linearity was confirmed by the test for lack of fit (P=0.653). The intra-assay and the inter-assay coefficients of variation (CV) for marbofloxacin were calculated to be 9.18 and 5.87, respectively.

Microbiological assay

Parallel to the HPLC determinations, the concentrations of marbofloxacin were measured by agar-gel diffusion method using *Escherichia coli* ATCC 25922 as test microorganism. The nutrient medium was meat-peptone agar (National Research Institute of Infectious and Parasitic Diseases, Sofia, Bulgaria). Standard solutions were prepared in serum obtained from untreated animals. The value of *r* for the standard curve was 0.993 and the linearity was confirmed by the test for lack of fit (P=0.749). The intra-assay CV was 9.09 and the inter-assay CV was 10.60. The limit of quantification in serum samples was 0.04 μg/ml.

Pharmacokinetic analysis

Pharmacokinetic analysis of the data was performed using non-compartmental analysis based on statistical moments theory (Gibaldi and Perrier, 1982) (WinNonlin 4.0.1., Pharsight Corporation, 800 West El Camino Real, Mountain View, CA, USA). The weighting scheme $1/y^2$ was used. The area under the serum concentration-time curve (AUC) was calculated by the trapezoid rule with extrapolation to infinity. The absolute bioavailability was calculated using the following equation:

(1) $F_{abs}\% = (AUC_{po} / AUC_{iv}) \times 100$.

Pharmacodynamic analysis

AUC/MIC and AUC/MBC values were obtained on the basis of the area under the concentration-time curve over 24 hrs divided by the MIC and MBC, respectively, which were determined in broth (Mouton *et al*., 2005). *Ex vivo* AUC/MIC and *ex vivo* AUC/MBC ratios were also determined. In these indices AUC (estimated by multiplying the measured serum concentration by incubation period of 24 hrs) was divided by MIC and MBC, respectively, as determined in serum. C_{max}/MIC and C_{max}/MBC values were estimated by using MIC and MBC values that were determined in serum (Aliabadi and Lees, 2001, 2002, 2003) and were used for PK-PD integration in this study. The log10 difference between the initial bacterial count (in number of CFU per millilitre) and the bacterial count after 24 h of incubation was also determined for turkey serum. To calculate the *ex vivo* AUC/MIC ratio in the effect compartment required for bacteriostic and bactericidal activities, and for the

total elimination of bacteria, the sigmoid inhibitory E_{max} model was used. Antibiotic response (expressed in terms of reduction of the initial bacterial count) is regressed against the surrogate marker (*ex vivo* AUC/MIC) using the Hill equation:

$$(2)\ E = E_{max}-[(E_{max}-E_0)\times C_e^N)/(EI_{50}^N + C_e^N)],$$

where E is the antibacterial effect measured as the change in the bacterial counts (in log10 CFU per millilitre) in the serum sample after 24 h of incubation compared to the initial log10 CFU per millilitre; E_{max} is the log10 difference in bacterial counts between 0 and 24 h in the control sample; E_0 is the log10 difference in bacterial counts in the test sample containing marbofloxacin after 24 h of incubation when the limit of detection of 10 CFU/ml is reached; EI_{50} is the *ex vivo* AUC/MIC producing 50% of the maximal antibacterial effect; C_e is the *ex vivo* AUC/MIC in the effect compartment (serum); and N is the Hill coefficient, which describes the steepness of the *ex vivo* AUC/MIC-effect curve. In these investigations, E_{max} represents the baseline bacterial count and E_0 is the maximal effect because the drug inhibits bacterial growth (Aliabadi and Lees, 2001, 2002; Aliabadi et al., 2003b). Hence the antibacterial response is the dependent variable representing the reduction of the initial bacterial count. The independent variable is the surrogate *ex vivo* AUC/MIC value. These PD indices were calculated on the basis of all samples by using the WinNonlin nonlinear regression program.

PK-PD analysis

By using *in vitro* MIC data and *in vivo* PK parameters, the surrogate markers of antimicrobial efficacy, AUC/MIC, C_{max}/MIC, and $T_{>MIC}$ were determined for serum after both, i.v. and oral administration of marbofloxacin. Because it is not possible to obtain large volumes of blood from turkeys, the PK-PD simulations were done on the basis of all values obtained from treated animals.

Antibacterial efficacy was quantified from the sigmoid E_{max} equation (Equation 2) by determining *ex vivo* AUC/MIC required for a bacteriostatic effect (no change in bacterial count after 24 h of incubation), a 50% reduction in the bacterial count, a bactericidal effect (a 99.9% decrease in the bacterial count), and for the bacterial elimination (the lowest *ex vivo* AUC/MIC that produced a reduction in bacterial counts to 10 CFU/ml) in serum (Aliabadi and Lees, 2001).

Statistical analyses

The pharmacokinetic parameters of marbofloxacin were presented as mean $\pm$ SD. They were computed with the Statistica 6.1 computer program (Statistica for Windows, StatSoft, Inc., USA, 1984-2002). A statistical analysis of the data obtained from the microbiological assays and from HPLC analysis was carried out using the Wilcoxon test. A value of $P<0.05$ was considered significant. The same program was used for statistical analysis of the standard curves.

Results

MIC and MBC values for marbofloxacin.

The MIC (0.125 μg/ml) and MBC (0.5 μg/ml) values in broth were 4-fold lower than values of MIC (0.5 μg/ml) and MBC (2.0 μg/ml) in serum.

Intravenous administration of marbofloxacin.

Data show the results of the HPLC determination, as there was no significant difference between HPLC results (Fig. 1) and the results from the microbiological assay (data not shown). A summary of the kinetic parameters is given in Table 1. The PK-PD integration index AUC/MIC resulting from *in vivo* kinetics and *in vitro* MIC in serum values for marbofloxacin was 23.58 (versus AUC/MIC of 94.32). These results indicate that the concentrations in serum exceed MIC values in serum (0.5 μg/ml) over a period of 9 hours.

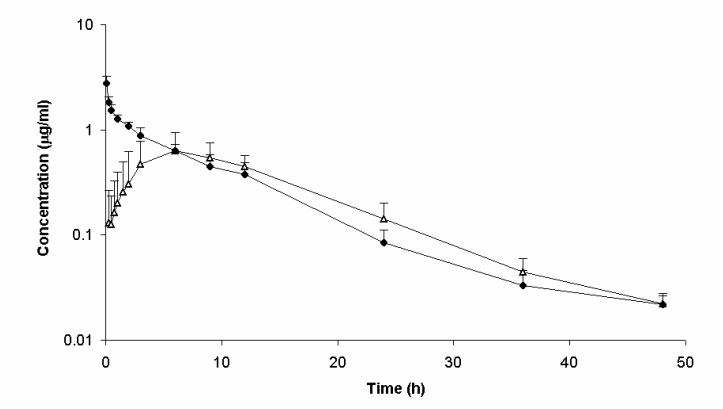

Fig. 1. Mean serum concentrations ± SD of marbofloxacin (at a dose of 2 mg/kg bw) after a single i.v. (♦) or oral (▲) administration in turkeys (n=6 animals).

Oral administration of marbofloxacin.

Peak serum concentrations were found between 3 and 6 h after drug administration, and the estimated MAT (mean absorption time) was 4.97±2.59 h. After 48 h the residual concentrations were close to the limit of quantification (Fig. 1 and Table 1). The AUC/MIC, was approximately 18 (18.4±6.4) and C_{max}/MIC was 1.34±0.58, calculated on the basis of MIC values in serum. The values of AUC/MIC (73.69±25.54) and C_{max}/MIC (5.35±2.31), were nearly 4 times higher, when MIC in broth was used. The value of $T_{>MIC}$ was 10.9 h.

Table 1. Pharmacokinetic parameters (non-compartmental analysis) of marbofloxacin in turkeys after i.v. and oral administration, respectively, of 2 mg marbofloxacin/kg bw. Data represent mean±SD of 6 individual animals.

Pharmacokinetic parameters	Units	HPLC analysis	Microbiological assay
Non-compartmental analysis – intravenous application			
$t_{1/2\beta}$	h	7.37 ±1.66	9.01 ±3.14
Cl_B	$ml.h^{-1}.kg^{-1}$	158.4 ±27.5	116.6 ±45.22
Vd_{area}	$l.kg^{-1}$	1.66 ±0.34	1.75 ±0.25
Vss	$l.kg^{-1}$	1.41 ±0.25	1.54 ±0.19
MRT	h	9.04 ±1.71	11.29 ±3.67
AUC_{0-24h}	$\mu g.h.ml^{-1}$	11.79 ±1.97	13.41 ±2.64
$AUC_{0-\infty}$	$\mu g.h.ml^{-1}$	12.94 ±2.21	16.71 ±5.36*
Non-compartmental analysis- oral application			
MRT	h	14.01 ±3.38	11.69 ±2.54
AUC_{0-24h}	$\mu g.h.ml^{-1}$	9.21 ±3.19	10.07 ±3.59
$AUC_{0-\infty}$	$\mu g.h.ml^{-1}$	10.89 ±3.21	10.86 ±3.45
$t_{1/2\beta}$	h	7.73 ±1.00	6.23 ±1.63
C_{max}	µg/ml	0.67 ±0.29	0.80 ±0.32
T_{max}	h	6.0 ±3.29	6.50 ±3.51
F_{abs}	%	84.37 ±21.26	70.67 ±30.66

$t_{1/2\beta}$ - terminal elimination half-life; $AUC_{0-\infty}$ - area under the serum concentration-time curves from 0 h to ∞; AUC_{0-24h} - area under the serum concentration-time curves from 0 h to 24 h; Vd_{area}, Vss - area volume of distribution, steady-state volume of distribution, respectively; MRT - mean residence time, Cl_B - total body clearance; C_{max} - maximum serum levels; T_{max} - time of C_{max}; F_{abs}% -absolute bioavailability.
* - Differences are statistically significant ($p<0.05$).

Antibacterial activity in serum of animals treated orally with marbofloxacin.

The activity of marbofloxacin against *E. coli* in serum of treated animals was determined and prominent inhibitory effect was observed for samples taken between 3 and 12 h, whereas at 24 and 36 h no significant inhibition of bacteria could be measured. The antibacterial time-dependent-killing curves are presented in Fig. 2. This figure presents with the control values (taken at 0 hours) the log-normal growth curve of the *E.coli* test strain in serum from untreated turkeys, that have to be compared with the bacterial growth curves in serum samples taken at the indicated time intervals after treatment.

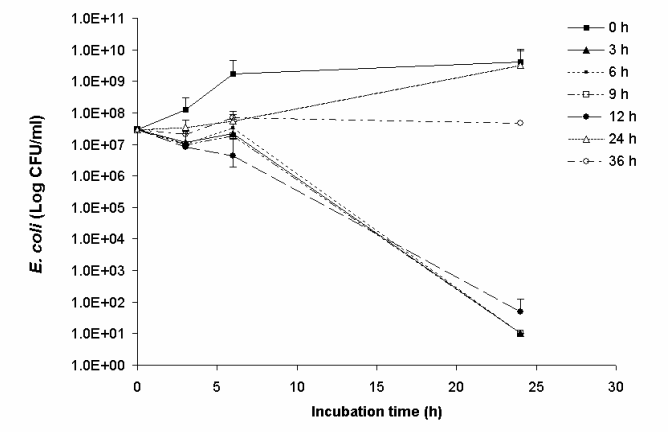

Fig. 2. Antibacterial activity (plots of log10 CFU per ml versus time) against *E. coli* O78/K80 in serum after oral administration of 2 mg/kg bw marbofloxacin. Values are means±SD (n=6).

Calculation of ex vivo AUC/MIC required for a bacteriostatic or bactericidal activity, and for total elimination of bacteria.

Graphs depicting the bacterial counts and *ex vivo* AUC/MIC relationships for serum for 24 h are presented in Fig. 3. The lowest *ex vivo* AUC/MIC required for bacterial elimination was lower than AUC/MIC. The steep slope of the *ex vivo* AUC/MIC-versus-bacterial count relationship explains the relatively similar values calculated for *ex vivo* AUC/MIC ratios that produced bacteriostatic or bactericidal activity (Table 2).

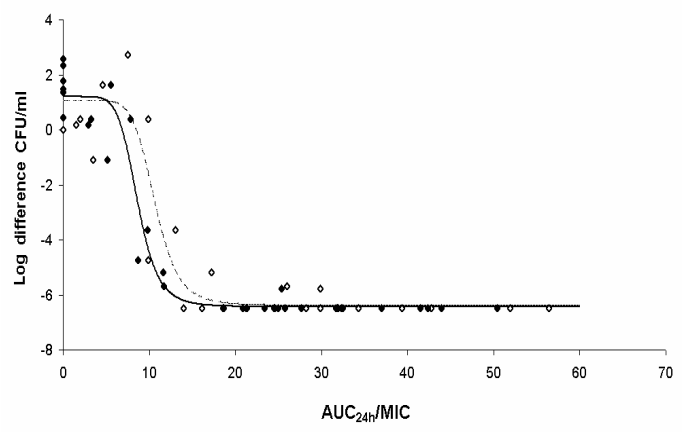

Fig. 3. Plots of *ex vivo* AUC/MIC versus bacterial count (log10 CFU per ml) for *E. coli* O78/K80 in serum of turkeys. The curve represents the line of predicted values, based on the sigmoid E_{max} equation and the points are the values of the individual animals (♦and — line – HPLC data; ◊ and - - line – microbiological data).

Table 2. Integration of pharmacokinetic and pharmacodynamic data obtained for marbofloxacin after oral administration of 2 mg/kg bw in turkeys (n=6).

Index	HPLC analysis	Microbiological assay
Log E_0 (CFU/ml)	-6.43	-6.37
Log E_{max} (CFU/ml)	1.20	1.08
EI_{50}	8.66	10.63
$LogE_{max}$-$LogE_0$	7.63	7.45
Slope (*N*)	7.28	7.84
ex vivo AUC/MIC		
Bacteriostatic	6.88	8.47
Bactericidal	8.90	10.89
Elimination	12.75	15.48

Log E_0 - difference in log of number of bacteria (CFU/ml) in sample incubated with marbofloxacin between time 0 and 24 h, when the detection limit (10 CFU/ml) is reached; Log E_{max} - difference in log of number of bacteria (CFU/ml) in control sample (absence of marbofloxacin) between time 0 and 24 h; EI_{50} (*ex vivo* AUC/MIC_{50}) - *ex vivo* AUC/MIC of drug producing 50% of the maximum antibacterial effect; *N* - the Hill coefficient; *ex vivo* AUC/MIC - ratio, required for bacteriostatic, bactericidal effect and bacterial elimination.

Discussion

Data on pharmacokinetics of marbofloxacin in poultry is limited, and specific pharmacokinetic data for turkeys are lacking. Hence, turkeys were treated with marbofloxacin at the recommended dose of 2 mg/kg bw, either by i.v. or by oral route. The serum concentrations were measured by two independent methods, a standardized HPLC method allowing the quantification of parent marbofloxacin, and a bioassay measuring antimicrobial activity in serum samples from treated animals. This microbiological assay would detect also any biologically active metabolites of marbofloxacin. Data show that the results obtained with both assays are very comparably. This good correlation indicates that the metabolism of marbofloxacin in turkeys is limited and suggests that any formed metabolite is less or not microbiologically active, which is in agreement with previous studies (Anadón *et al*., 2002).

Following i.v. injection, the value of $t_{1/2\beta}$ of marbofloxacin was longer in turkeys than in broilers (5.26 h) and buzzards (4.11 h) (Garcia-Montijano *et al.*, 2001; Anadón *et al*., 2002). In comparison with other fluoroquinolones (enrofloxacin, danofloxacin, fleroxacin, ofloxacin), marbofloxacin has a lower volume of distribution and a longer elimination half-life (Anadón *et al.*, 1997; Anton *et al.*, 1997; Liu and Fung, 1997; Knoll *et al*., 1999; Aliabadi and Lees, 2002). The calculated mean absorption time (MAT) suggests a rather slow absorption of the drug after oral administration, but the calculated bioavailability indicates a high rate of absorption (F=84.4%). In chickens, marbofloxacin was absorbed to a lower extent (F=56.8%), but C_{max} was detected earlier (Anadón *et al*., 2002). In comparison to danofloxacin (F=78.4%) and enrofloxacin (F=69.85%),

marbofloxacin has a higher oral bioavailability (Haritova *et al.*, 2004; 2006). The oxadiazine cycle in the molecule of marbofloxacin, which makes it different from other fluoroquinolones, seems to determine the higher oral bioavailability and the increased elimination half-life. In other studies with marbofloxacin in various animal species it was concluded that the pharmacokinetic properties of marbofloxacin seems to be advantageous as compared to over other fluoroquinolones (Anadón *et al*., 1997; Anton *et al*., 1997; Liu and Fung, 1997; Knoll *et al*., 1999; Aliabadi and Lees, 2002).

The most frequently used pharmacodynamic index for measuring the activity of an antimicrobial *in vitro* is the estimation of the MIC, and this value is used to predict the antimicrobial efficacy and potency of a drug. Although, MIC values in broth and in serum are comparable to published data for MIC_{90} values of most pathogenic *E. coli* strains (Spreng *et al*.,1995; Anadón *et al*., 2002), it should be reiterated that growth curves (and MIC values) measured in broth only, are less representative than that determined in serum or even *in vivo* findings. Our finding that in the presence of serum, the MIC values was reduced (resulting in values that exceeded the MIC in standard broth by approximately a factor of 4) coincides with previously reported data on the decreased antimicrobial activity of most fluoroquinolones in the presence of serum (2-4 fold higher MIC values; Jacobs *et al*., 2002; Aliabadi *et al*., 2003a, 2003b; Wise, 2003; Haritova *et al*., 2004). Protein binding explains the lower inhibitory activity of some fluoroquinolones in serum (Wise, 2003), but compared to other fluoroquinolones marbofloxacin has a rather low rate of plasma protein binding hence other factors may contribute as well to the observed differences (Mouton and Vinks, 2005).

PK-PD indices in the current study were used according to the standardized terminology and other terms were defined when these indices differ from the generally accepted definitions (Mouton *et al*., 2005). Clinical investigations in human medicine and animal studies have shown that AUC/MIC and C_{max}/MIC correlates strongly with the clinical response to fluoroquinolones with better predictive value of the first ratio (Schentag *et al*., 2001; Palladino and Callen, 2003). The calculated values of C_{max}/MIC for marbofloxacin (1.34 – 1.58) were lower than the comparable values for danofloxacin mesylate (4.06) for the investigated strain *E.coli* O78/K80 (Haritova *et al*., 2006), reflecting the lower potency of marbofloxacin. For danofloxacin the C_{max}/MIC ratio obtained with the recommended therapeutic dose (6 mg/kg bw, orally) results in a 99% reduction in bacterial counts (Drusano, 2000; Scaglione, 2002; Haritova *et al*., 2006). The results presented here for marbofloxacin and previously published data for enrofloxacin in turkeys (C_{max}/MIC - 1.7) suggest a higher survival rate of pathogens, hence indicating a risk for development of antimicrobial resistance against fluoroquinolones in turkeys (EMEA, 1999; Giraud *et al*., 2001; Haritova *et al*., 2004).

The steep slope of *ex vivo* AUC/MIC versus bacterial count curves with a high Hill coefficient and *in vitro* investigations demonstrate that marbofloxacin, like danofloxacin, exerts a concentration-dependent killing against different strains of *E. coli* (Toutain *et al*., 2002; Schneider *et al*., 2004). However, the antibacterial activity of marbofloxacin against

E. coli O78/K80 in serum (determined as log10 CFU/ml difference in bacterial count in the test sample containing marbofloxacin) appeared to be slower during the first six hours of incubation, as compared to danofloxacin (Haritova *et al.*, 2006, see Chapter 2). Bacterial elimination could be achieved with danofloxacin at lower *ex vivo* AUC/MIC ratios in comparison to marbofloxacin (Haritova *et al.*, 2006). Marbofloxacin, however, possess some preferable pharmacokinetic properties in comparison to other fluoroquinolones such as low serum protein binding and Cl_B, which should compensate for the lower activity against *E. coli* O78/K80 (Haritova *et al.*, 2004; 2006).

Applying the integrated PK and PD approach, estimated surrogates and using the equation proposed by Toutain *et al.* (2002) (Dose=(AUC/MIC×Cl_B×MIC)/F) the calculated dose for marbofloxacin equals 1.2 mg/kg bw per 24 h. Considering also the AUC/MIC value (18.42 h), calculated on the basis of MIC in serum, achieved with the recommended dose for marbofloxacin of 2 mg/kg, it can be assumed that this fluoroquinolone could be appropriate choice to achieve clinical cure of *E. coli* infections. A remaining limiting variable is the varying intrinsic sensitivity of field isolates of *E. coli* against marbofloxin, as in our approach the PD data (i.e. MIC and MBC values) were determined only in one individual strain. McKellar *et al.* (2004) suggested incorporating MIC_{90} and MIC values from one strain in the PK-PD calculation as indicative for the variability of *E. coli* isolates. A prerequisite, however, is the availability of representative data in this case from different *E. coli* strains isolated from turkeys. Other factors which also could influence the outcome of treatment such as immunity status of birds, physiological changes during infection, tissue distribution of drug are not considered in the PK-PD modeling.

The therapeutic use of fluoroquinolones in poultry is only assessed in terms of good clinical efficacy, but needs to consider the risk of the induction of antimicrobial resistance, as zoonotic pathogens like *Salmonella spp.* and *Campylobacter spp* are prevalent in poultry flocks and can be transmitted via meats to consumers (Fiorentin *et al.*, 2003). Gunderson *et al.* (2001) and Hyatt *et al.* (1994) recommended that for the treatment of Gram-negative infections higher AUC/MIC ratios, along with high values for C_{max}/MIC and $T_{>MIC}$ surrogates, should be used to reduce the risk of resistance induction. These authors recommend a breakpoint of 125 (AUC/MIC > 125) to reduce the risk of emergence of resistance (Gunderson *et al.*, 2001; Schentag *et al.*, 2001; Wise, 2003).

Following the paradigm that the AUC/MIC ratio should exceed a value of 125, reaching under optimal conditions even a ratio of 400-500 (Hyatt *et al.*, 1994), the data presented would suggest optimal doses of 3.0 up to 12.0 mg/kg bw per day for marbofloxacin if MIC is 0.125 μg/ml. This suggestion is in line with the proposed higher dose regimens for danofloxacin, enrofloxacin, sarafloxacin and norfloxacin in turkeys (Laczay *et al.*, 1998; Haritova *et al.*, 2004; 2006). As was already mentioned above, the applied approach has limitations since the activity of marbofloxacin was not determined here in challenge experiments, and PK-PD indices serve as surrogate markers for efficacy. Therefore, clinical trials should validate this dose in diseased turkey flocks under practical

conditions, assessing not only bacteriological cure rates, but also monitor the emerge of antibacterial resistance.

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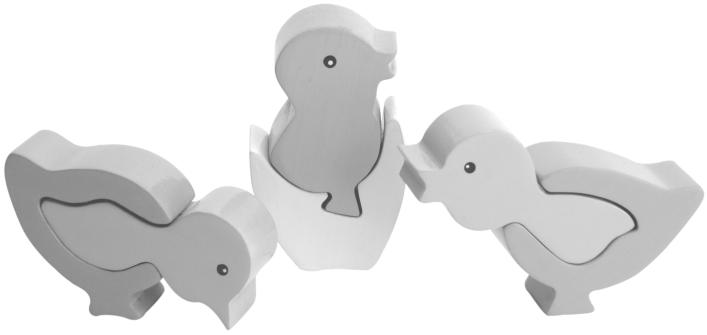
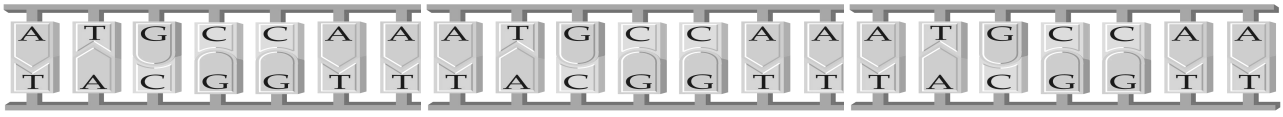
CHAPTER 4





4

Implantation of Tissue Chambers in Turkeys: A Pilot Study

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Introduction

Tissue chambers are used as a model to study the composition of the interstitial fluid since 1963 (Guyton, 1963). Under experimental conditions, tissue chambers have been placed in the peritoneal cavity or in the subcutaneous space, where it remains accessible for transcutaneous punctures allowing to obtain in parallel serum/plasma samples and tissue fluid. A revival of this technique occurred in 1987, when Lees and co-workers re-established the model as non-invasive tool to study the local inflammatory response and drug concentrations of anti-inflammatory drugs as the site of action (Higgins *et al.*, 1987; Lees *et al*., 1987). Compounds such as carrageenan and lypopolysaccharide (LPS) were used to provoke a local inflammatory reaction. Parameters studied in the tissue cage fluid included inflammatory mediators (cytokines, eucosanoids), leukocyte influx and skin temperature over the tissue chamber by serial measurements (Higgins *et al.*, 1984; Higgins *et al*., 1987). Moreover, drug penetration into inflamed (exudate) and non-inflamed (transudate) chamber fluid was measured as a surrogate for the distribution of the drug over the interstitial tissue space (Onderdonk *et al.*, 1989; Vogel *et al.*, 1996; Erlendsdottir *et al*., 2001; Liu *et al*., 2002, Sidhu *et al*., 2003).

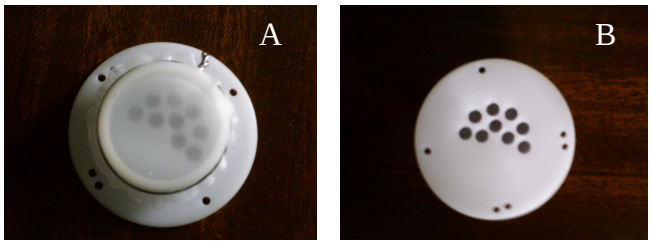
More recently, essentially the same technique was applied to study pharmacokinetic/pharmacodynamic (PK-PD) interactions of antimicrobials measuring not only drug concentrations in the tissue chamber, but also the effect of the therapeutic efficacy of animicrobials against local infections with diverse pathogens (Greko *et al*., 2003; Aliabadi and Lees, 2001; 2002; 2003; Aliabadi *et al.*, 2003).

Whereas the tissue cage model has been validated in various mammalian species, experiments in poultry are lacking.

Materials and Methods

A pilot experiment was conducted to evaluate the possibility to implant tissue chambers in birds. To this end, a one-year-old healthy female turkey, BUT 9 breed, 5.15 kg body weight, was selected. It was given free access to commercial food for turkeys (without antibacterials and coccidiostatics) and the animal was kept with other turkeys in a box stand.

A round custom-made tissue chamber was used for implantation (see picture 1). It



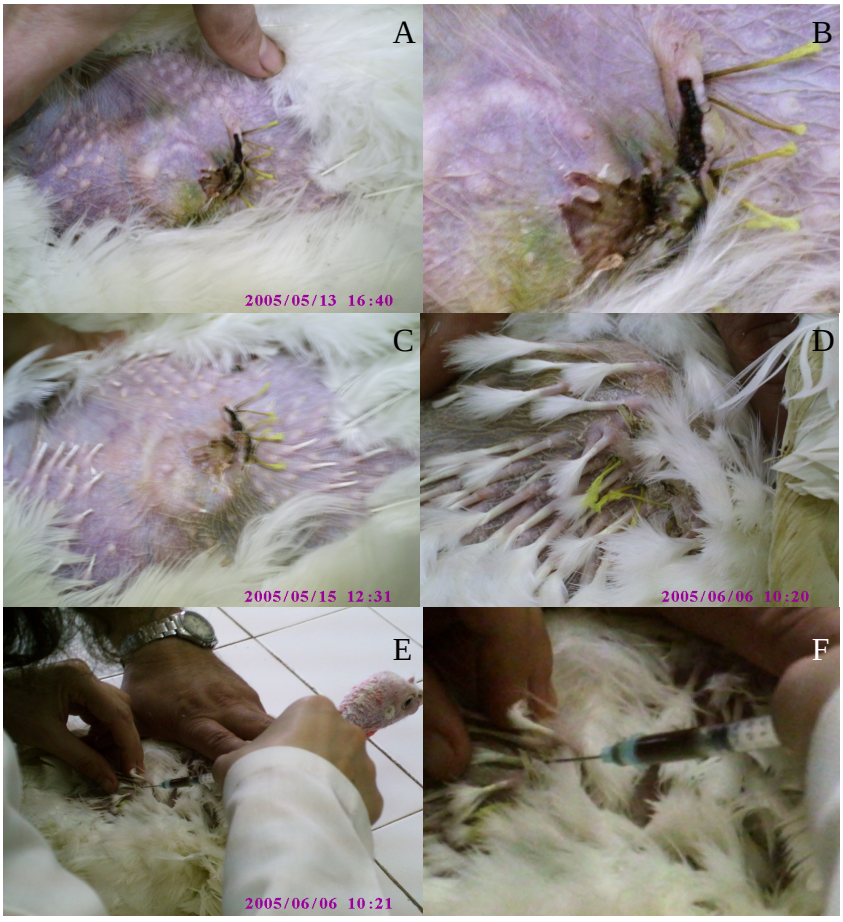
Picture 1. Tissue chamber. A – upper side with membrane and B – bottom side.

had an inner diameter of 2.2 cm and a depth of 1 cm and contained 9 holes in the bottom and 12 holes on the side surface. The total volume of the empty chamber was 2.2 ml.

The cage was aseptically implanted subcutaneously under the right wing, above the *M. pectoralis thoracicus.* After implantation, the animal was allowed to recover for a period of 4 weeks.

Results and Discussion

Pictures 2A and 2B show the implanted tissue cage on 4th day after surgery. Picture 2C and 2D were taken 10 days and 29 days after implantation, respectively, demonstrating



Picture 2. Implanted tissue cage. A and B show the implanted tissue cage on 4th day after surgery; C and D - 10 and 29 days after implantation, respectively. E and F – puncture of the cage on 29th day.

that the tissue chamber was implanted successfully without visual signs of inflammation. Feathers reappeared on the skin-surface at the end of the experiment (Picture 2D). On day 29 p.i. attempts to aspirate tissue cage fluid were made at times zero, one and three hours, and at each time point 0.5 ml fluid could be withdrawn. When after 24 hrs the 4th sampling was conducted only 0.2 ml fluid could be aspirated. It should be mentioned that the withdrawn tissue fluid was contaminated with blood, which might be a problem in experiments in which drug concentrations should be measured in the tissue chamber fluid in parallel with blood serum/plasma samples.

The obtained results from this pilot experiment also indicate the limitations in the amount of tissue fluid that can be obtained from the chamber in serial experiments. After removal of the cage on day 40 after implantation approximately 60% of the internal volume was filled with connective tissue, which explains the limited fluid volume. Previous experiments in mammals had already indicated that the size and shape of tissue chambers and the number and size of holes influence the composition and rate of formation of tissue cage fluid (Bergan, 1981). Moreover, the age of the tissue chamber influences the amount of tissue fluid produced upon a challenge (Aliabadi and Lees, 2001).

In conclusion, this first pilot experiment suggests the possibility to use tissue chambers also in turkeys or other avian species. Special small and tailor-made chambers are necessary according the size of the animals. Further experiments need to be conducted to assess the most optimal time points at which tissue chamber fluid can be withdrawn at regular intervals and to identify agents that result in a reproducible local inflammatory response (Roacha and Sufka, 2003). These experiments will provide valuable details regarding the inflammatory response in terms of cellular infiltration and the production of inflammatory mediators in avian species, and allow the assessment of the efficacy of anti-inflammatory agents as well as antimicrobials in the interstitial space.

Acknowledgements

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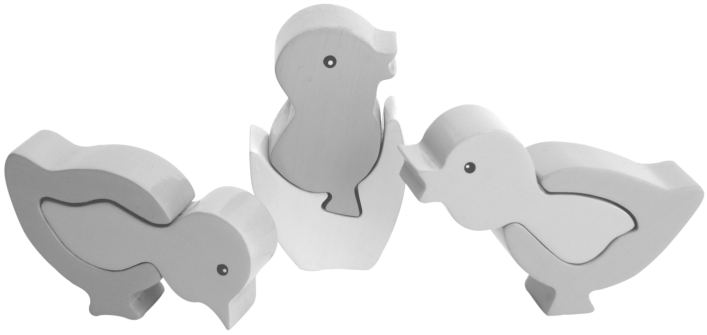
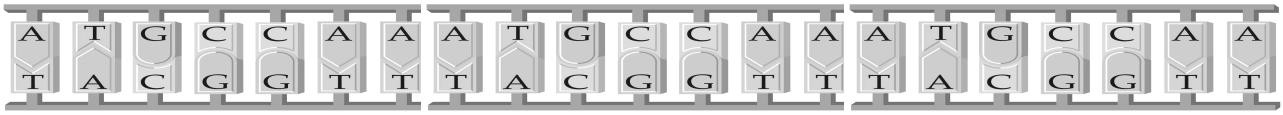
CHAPTER 5





5

Expression of Drug Efflux Transporters in Poultry Tissues

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Based on a manuscript submitted to Poultry Scienece

Abstract

Efflux transporters play an important role in the absorption, distribution and excretion of drugs and toxins. Multidrug resistance 1 (MDR1) and multidrug resistance-associated protein 2 (MRP2) encode two prominent members of the family of transmembrane proteins that function as efflux transporters. Both belong to the family of adenosine triphosphate (ATP)-binding cassette transporters that can carry a wide range of substrates, including physiological substances as well as drugs and toxins across biological membranes using ATP as energy source. The level of expression of these transporters in different tissues has hitherto been studied mainly in mammals, and only P-glycoprotein (P-gp), the product of the MDR1 gene, has been described in chickens as yet. Therefore, the aim of this study is to describe the level of expression of MDR1 and MRP2 in different tissues of chickens.

Tissue samples from eight six-week-old Isa-brown chickens of the crop, proventriculus, duodenum, proximal and distal jejunum, ileum, caecum, colon, liver, kidney, lung, brains, adrenal glands and testes were collected and submitted to real-time (RT)-PCR analysis. The levels of mRNA expression of MDR1 and MRP2 were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin.

The highest MDR1 expression was found in the ileum, followed by the proximal and distal jejunum, duodenum, liver, colon, kidney and lungs. A relatively low level of expression was noticed in the adrenal glands, brains, proventriculus and caeca, and an even lower expression was measured in the crop and the immature testicles and ovaries. The MRP2 expression exceeded that of MDR1 in the liver and kidney. A high MRP2 mRNA expression was also detected in the duodenum and the proximal and distal jejunum, while expression was low in the ileum and the lungs. Comparing the findings in chickens with previously published data, in particularly those from humans and rodents, a high degree of similarity in the expression of MDR1 and MRP2 was apparent, suggesting a comparable function of these transporters in chickens.

Introduction

Kinetic parameters such as rate of absorption after oral administration, distribution of a drug within the body and drug concentrations at target sites, as well as the routes and rate of excretion are determining the residence time of any drug in the animal's (or human) body and are major determinants of the dose regime that needs to be applied to achieve clinical efficacy. Increasing evidence suggest that specific drug transporters, that use ATP as an energy source to actively transport their substrates across biological membranes, denoted ABC transporters, affect all these kinetic parameters (Kusuhara and Sugiyama, 2002). Subsequently, these ABC transporters were found to play also an important role in the physiological homeostasis of an organism, as they modulate not only the fate of xenobiotics but also determine the intracellular concentration of numerous endogenous substrates, including neurotransmitters, signalling molecules and hormones (Dean and Annilo, 2005). The clinically most relevant effect is the contribution of these efflux transporters to functional tissues barriers, such as the blood-brain/testes-barrier, and in mammals also to the placental barrier as well as the blood milk barrier.

The contribution of efflux proteins to biological barriers implicates their role as determinants of oral bioavailability and subsequent tissue distribution and elimination of drugs and toxins, as they are expressed at apical membranes in the intestines, liver and kidney, as first described by Thiebaut *et al.* (1987). Both P-glycoprotein (**P-gp**) and multidrug resistance-associated protein 2 (**MRP2**) are present in the biliary canalicular membrane of hepatocytes and facilitate the excretion of xenobiotics, including their glutathione and glucuronide conjugates. In the kidneys, these efflux transporters have been found in the brush border membranes of the (proximal) tubule epithelial cell and fulfil a similar role by pumping their substrates into the tubular lumen (Fardel *et al.*, 2005).

In contrast to humans and common laboratory animal species, the knowledge regarding the expression or function in other animal species, particularly in poultry is very limited. Therefore, we here describe the levels of expression of the mRNA of two prominent ABC transporters, multidrug resistance 1 (**MDR1**) and MRP2 in different organs of chickens. Organ samples were collected from healthy chickens, and subjected to real-time (**RT**)-PCR analysis.

Materials and methods

Animals

Eight Isa-Brown chickens, 6-weeks old, of both sexes (4 male and 4 female) were selected. The female chickens had a body weight of 510-580 grams, and the males of 600-710 grams. Prior to the experiments, the animals had been kept under the normal conditions of a poultry farm and had been subjected to the standard vaccination program. Birds were given access to feed (2,760 kcal ME/kg, 17% CP) and water *ad libitum*. Clinical examination confirmed the absence of any manifest diseases at the time of the experiment.

After euthanasia by electric shock, tissue samples of the following organs were quickly removed and snap-frozen in liquid nitrogen: crop, proventriculus, duodenum, proximal and distal jejunum, ileum, caecum, colon, liver, kidney, lung, brain, adrenal glands and testis or ovaries. Samples were stored at -70°C until analysis.

RNA isolation

Total RNA was isolated using TRIzol Reagent (Cat. No. 15596-018, Invitrogen Life Science Technologies). Approximately 50 to 90 mg of each tissue sample was mechanically homogenized using an Ultra Thurrax (Janke & Kunkel, IKA Labortechnik) in 1.0 ml of Trizol. After centrifugation and incubation for 5 min at room temperature, 0.2 ml chloroform was added and the mixture homogenized. After centrifugation at 12.000 *x g* for 15 min, the aqueous (upper) layer containing the RNA was harvested, and total RNA was precipitated with 0.5 ml isopropanol. After precipitation, the vials were centrifuged again at 12.000 *x g* for 10 min. The supernatant was removed and the resulting pellet washed with 1.0 ml of 75% ethanol and centrifuged again at 7.400 *x g* for 5 min. The supernatant was discarded and the residual ethanol was evaporated and the obtained pellet was subsequently re-suspended in RNAse-free water. The concentration of RNA was quantified by spectrophotometry at 260 nm (ND-1000, Nanodrop technologies). Samples were stored at -70^{o}C until further processing.

cDNA synthesis

First strand cDNA was synthesized using the iScript^{tm}cDNA Synthesis Kit (*Bio-Rad* Laboratories, USA) according to the manufacturer's instructions. A final volume of 20µl, containing 1 µg of the total RNA, iScript Reaction Mix and iScript reverse transcriptase was incubated for 5 min at 25^{o}C, then at 42 ^{o}C for 45 min, followed by heat inactivation of the enzyme at 85^{o}C for 5 min and a final fast cooling step. cDNA samples were stored at -20^{o}C.

Real-time PCR analysis

Specific primers for the chicken homologues to MDR1 and MRP2, glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) and beta-actin were designed and commercially produced (Isogen Bioscience BV, The Netherlands). The efficiency of the primers and the optimal annealing temperatures were evaluated by the qPCR analysis of a dilution series of mixed cDNA samples, run in a PCR cycle with a temperature gradient. The specificity of the primers was evaluated by melting curve analysis, agarose gel electrophoresis and subsequent sequence analysis (data not shown). The final primers used (Table 1) were all specific for their targets and highly efficient (efficiency > 95%).

Table 1. PCR primers for the quantitative PCR analysis.

Gene	NCBI accession number	Forward primer 5' ® 3'	Reverse primer 5' ® 3'	Tm ^{o}C
MDR1	NM_204894	GCTGTTGTATTTCCTGCTATGG	ACAAACAAGTGGGCTGCTG	58
MRP2	XM_421698	CTGCAGCAAAATGAGAGGACAATG	CAGAAGCGCAGAGAAGAAGACCAC	63
GAPDH	NM_204305	GTGTGCCAACCCCCAATGTCTCT	GCAGCAGCCTTCACTACCCTCT	65
Beta-actin	NM_205518	ATGTGGATCAGCAAGCAGGAGTA	TTTATGCGCATTTATGGGTTTTGT	61

MDR1 - multidrug resistance 1; MRP2 - multidrug resistance-associated protein 2; GAPDH - glyceraldehyde 3-phosphate dehydrogenase; NCBI – The National Center for Biotechnology Information; Tm – optimal annealing temperature.

The PCR analysis was performed with iQTM SYBR Green Supermix (Cat. No. 170-8885, Bio Rad Laboratories Inc.), conducted according to the instructions of the manufacturer. Each reaction mixture contained 15µl supermix, forward and reverse primers (10 pmol each), and 50 ng reverse-transcribed RNA (1µl). The samples were processed in an iCycler iQ PCR system (Bio-Rad, Hercules, CA) and analysed using MyiQ System Software, Version 1.0.410 (Bio Rad Laboratories Inc.).

Following an initial hot-start for 3 min, each reaction went through a PCR cycle with a denaturation step at 95^{o}C for 20 s, an annealing step specific for each set of primers for 30 s and an elongation step at 72^{o}C for 30 s. After 35 cycles a melting curve was obtained by increasing the temperature with 0.5^{o}C every 10 s from 65^{o}C to 95^{o}C.

A dilution series of cDNA form mixed samples was included in each 96-well PCR-plate as a control for the PCR reaction. All analyses were done in duplicate.

Gene expression was presented using the algorithms outlined by Vandesompele *et al*. (2002) and the geNorm manual available on the web site http://medgen.ugent.be/~jvdesomp/genorm/. Dilution series of target and reference nucleic acids were used to determine the fit coefficients of the relative standard curve (efficiencies).

Statistical analysis

The results are presented as mean ± SD. All data were analysed with the Statistica 6.1 computer program (Statistica for Windows, StatSoft, Inc., 1984-2002). Statistical analysis was done with ANOVA and the Tukey post-hoc test. The parametric method was chosen after the test for Gaussian distribution.

Results

All tissues expressed measurable mRNA concentrations of housekeeping genes GAPDH and beta-actin, with similar values of threshold cycle (**C_t value**). MDR1 mRNA expression was detected in all samples. The highest MDR1 mRNA expression was observed in the ileum, followed by the proximal and distal jejunum, duodenum, liver, colon, kidney and lung. A relatively low expression was observed in the adrenal glands, the brain, proventriculus and the caeca; and even lower expression was found in the crop, and the immature testes and ovaries of these young animals.

The MRP2 mRNA expression exceeded that of MDR1 in the liver and the kidney. High MRP2 mRNA expression was detected also in the duodenum and proximal and distal jejunum, decreasing in the ileum (Figure 1). Low levels were also found in lung tissues. In some organs such as crop, proventriculus, caeca, colon, brain and the adrenal glands, the levels of expression of MRP2, however, were too low to be quantified (data not shown).

The inter-individual variability in expression levels was relatively high, and higher in the mRNA levels of MRP2 as compared to MDR1 mRNA. A statistical comparison of the MDR1 mRNA expression levels in the different parts of the gastro-intestinal tract revealed that MDR1 mRNA expression in the small intestine significantly exceeded that of the large intestine ($P<0.001$). In the crop and proventriculus MDR1 levels were significantly lower than those found in the small intestine, colon and in the liver. The highest ($P<0.001$) expression levels were found in the ileum. After a significant decrease in the caeca the levels increased again in the colon (see Figure 1). The MRP2 mRNA expression was higher in the liver than in the small intestines ($P<0.001$). In contrast to MDR1, MRP2 mRNA expression decreases along the intestinal tract, and in the caeca no measurable expression could be found.

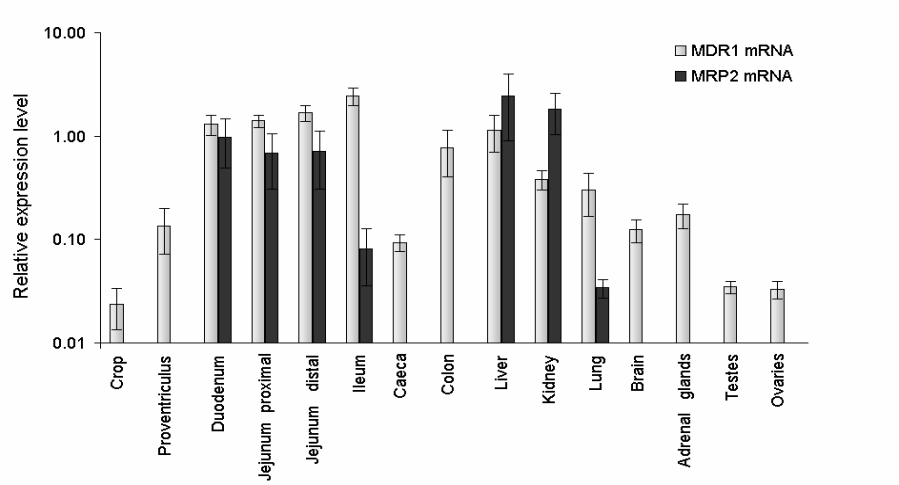

Figure 1. Relative levels of expression of multidrug resistance 1 (MDR1) and multidrug resistance-associated protein 2 (MRP2) mRNA normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin.

Discussion

The expression of genes encoding for ABC transporters has been analysed predominantly in rodents as well as in human tissue samples, whereas data in farm animal species are almost completely lacking. Edelmann *et al.* (1999) and Barnes (2001) had described the expression and function of chicken P-gp by Northern and Western blot analysis. They demonstrated the presence of mRNA and the protein in the liver, but also in

other tissues such as the intestinal tract, kidneys and lungs. Functional characterization demonstrated a high similarity with human P-gp, and the protein sequence alignments showed a nearly equal homology to both human MDR1 (P-gp) and human MDR3. During the preparation of this manuscript the original cDNA sequence for chicken P-gp (NCBI accession number GGA9799) introduced by Edelmann *et al.* (1999), was re-evaluated by the NCBI and designated ABCB4 based on sequence similarity. The ABCB4 gene encodes for the highly related human phosphatidylcholine transporter MDR3 P-glycoprotein, with a detectable protein expression only in the (human) liver (Smit *et al.*, 1994; Oude Elferink and Paulusma, 2006). It is noteworthy to mention in this context that the variation for human–chicken alignments (sequence conservation - median amino acid identity of 75.3%) is much greater than the variation seen for human–rodent alignments (88%) (International Chicken Polymorphism Map Consortium, 2004). Therefore, in the absence of further functional studies and considering that the protein-expression data indicate that the cDNA encodes a chicken P-gp homologue in the forthcoming text only the term MDR1 is used, to allow an easy comparison with comparable studies in mammalian species.

The presented data give for the first time an indication about the levels of expression of two important ABC transporters in various tissues of chickens. To allow an overall comparison of the differences and similarities in the expression patterns between chickens (as representatives of avian species) and mammals, the available data are discussed per organ system:

Gastrointestinal tract

The upper part of the gastrointestinal tract is unique in birds, and crop and proventriculus have no direct equivalent in mammals. MDR1 mRNA expression levels at both locations were found to be very similar, but low in comparison with other parts of the gastrointestinal tract. The increasing MDR1 mRNA levels from the duodenum to the ileum, found in the presented study, are in line with the observed abundance of this mRNA in the small intestines in mammals. Also the increasing presence of MDR1 mRNA in the colon of poultry is similar to that in mammals. High MDR1 mRNA levels in the small intestine can be related to its physiological function as a place for nutrient absorption, and the exposure to non-nutritional feed components and toxins. This protective function of P-gp as part of the intestinal barrier has been recognized in humans, rodents and pigs, and hence seem to apply also to avian species (Tang *et al.*, 2004; Thörn *et al.*, 2005).

As mentioned above, only very limited data on the level of expression of MDR-genes in chickens are available from previous studies. All these data were obtained with Northern blotting and suggested that a high MDR1 expression has to be expected in the jejunum and ileum, but low levels in the rectum (Edelmann *et al.*, 1999). The corresponding protein levels (measured by Western blotting) were particularly high in the small intestines (duodenum, jejunum, and ileum showing similar expression levels) and in the caeca. In contrast, the expression levels in the proventriculus and colon were five-fold lower than those in the duodenum (Barnes, 2001). Our results with RT-PCR analysis of

MDR1 mRNA expression along the gastrointestinal tract in chickens confirm many of these previous results, with the exception of the caeca in which we found a low level of expression.

MRP2 mRNA levels along the gastrointestinal tract were found to be high in the duodenum and jejunum of rats, but low in the stomach, and even lower in the ileum and colon (Mottino *et al*., 2000; Rost *et al*., 2002). MRP2 expression in mice is particularly high in the small intestines, and it decreases from the duodenum to the ileum (Maher *et al.*, 2005). In humans, MRP2 has the highest expression in the jejunum, whereas lower levels were found in the duodenum. Even lower levels were found in the terminal ileum and the entire colon, where the expression decreased to almost non-measurable levels (Taipalensuu *et al.*, 2001; Zimmermann *et al.*, 2005). No data of chickens or other poultry species have been published as yet, but the results presented here show a high degree of similarity between the expression of MRP2 in chickens as representative of avian species, and the data found in mammals.

When comparing the levels of expression of MDR1 with that of MRP2, it seems that MDR exceeds the level of MRP2 in the duodenum and ileum of humans and chickens. In contrast to humans, in the chicken all parts of the small intestine express MRP2 mRNA at a lower level than MDR1, suggesting a higher importance for P-gp as an efflux pump in these animal species.

Liver

In the livers of chickens, high levels of MDR1 mRNA and functional P-gp protein had been detected previously (Edelmann *et al*., 1999; Barnes, 2001). In contrast, in mice expression of *mdr1a* in the liver accounts for less than 1/10 of that found in the ileum (Brady *et al*., 2002). The data presented here are in line with the previous results from poultry, as the expression in the liver seems to be only slightly lower than that in the small intestines. MRP2 is highly expressed in the hepatocyte canalicular membrane in the liver of rodents (Faber *et al*., 2003; Maher *et al.*, 2005). The results of the present study show that the MRP2 expression in the poultry liver exceeds those of MDR1 mRNA. MRP2 transports many conjugated substrates such as glucuronides or glutathione conjugates into the bile canicula, a function that is essential for both, mammalian and avian species.

Kidney

The *mdr1a* mRNA expression levels in mice are lower in the kidney than in for example the ileum, where moderate expression levels are seen (Brady *et al*., 2002). In humans, high levels of MDR1 mRNA and subsequently P-gp were found in the apical surface of the epithelial cells of the proximal tubules (Thiebaut *et al.*, 1987; Langmann *et al*., 2003). Similar results had been observed in chickens (Edelmann *et al*., 1999; Barnes, 2001) and our results confirm these previous findings. MRP2 expression has been localized in the brush-border membrane of proximal tubule cells of the kidneys of mice and humans (reviewed by Keppler *et al*., 1998) and we found comparable results in chickens.

Lungs

In humans, P-gp (MDR1) is expressed at the apical side of the ciliated epithelial cells or at the ciliated collecting ducts. Its expression is also seen on the apical and lateral surfaces of secretory cells of bronchial glands, tracheal epithelial cells and major bronchi (Lechapt-Zalcman *et al.*, 1997). A high MDR1 expression and an abundant P-gp level have been found in the lungs of poultry, using Northern and Western blotting (Edelmann *et al.*, 1999; Barnes, 2001). The presented results confirmed the presence of MDR1 mRNA in lung tissue at levels that are higher than those found for MRP2. Low MRP2 expression is also described in lungs of rats (Cherrington *et al.*, 2002) where it shows the same subcellular distribution as in humans i.e. major levels in bronchial epithelial cells and peripheral lung cells.

Brain

Investigations in human tissues indicated that P-gp expression is confined to the endothelial cells of capillary blood vessels and to the neuronal layer of the dentate gyrus, whereas it is absent in the cerebral cortex, cerebellum, and spinal cord (Thiebaut *et al.*, 1987; Karssen *et al.*, 2004). Using Northern blotting, MDR1 mRNA levels had been found in the poultry brain tissue (Edelmann *et al.*, 1999). In the present study, the expression of MDR1 in brain homogenates was confirmed. As total tissue homogentes were used, it can however not be excluded, that also in poultry P-gp expression is confined to the capillary endothelia of the central nervous system. The prominent contribution of P-gp to the function of the blood-brain barrier has been addressed in many reviews, and genetic defects in P-gp expression explain the sensitivity of individual animals (including certain breeds of dogs) towards potentially neurotoxic compounds like ivermectin (Geyer *et al.*, 2005).

MRP2 had been found in the apical membranes of capillary endothelium of the brain choroid plexuses in other animals like rats, pigs and humans (Potschka *et al.*, 2003), but remained below the detection limit in the total brain homogenates used in the presented experiments with chickens.

Adrenal glands

According to Brown *et al.* (1993) the adrenal glands do not express *mdr1b* mRNA in rats. In contrast, Langmann *et al.* (2003) describe that the levels of MDR1 expression in the human adrenal gland are amongst the highest in the entire body. This is in line with the high P-gp levels found in the adrenal medulla and cortex (Thiebaut *et al.*, 1987). RT-PCR analysis for MDR1 mRNA demonstrated the expression of MDR1 in the adrenals, albeit at a low level. In contrast, Northern blotting failed to detect MDR1 in chicken's adrenal glands (Edelmann *et al.*, 1999). As corticosteroids are substrates for P-gp, it is likely that P-gp (and probably other transporters) are involved in the hormonal homeostasis in avian species.

Testes and ovaries

MDR1 mRNAs and P-gp are found in the capillaries of the testes, interstitial cells and late spermatids in the mouse, rat and man (Melaine *et al*., 2002). The MDR1 levels of expression are classified as medium in the humans (Langmann *et al*., 2003). In contrast, no significant P-gp activity was found in human ovaries (Thiebaut *et al*., 1987). Using Northern blotting, Edelmann *et al*. (1999) could identify MDR1 mRNA levels in the ovarian follicles in poultry. Also our findings indicated a very limited MDR1 expression. It has to be noted, however, that in the present study juvenile animals were used and that mRNA levels might increase upon sexual maturation.

In conclusion, the presented data summarizing for the first time the levels of the expression of the two major ABC transporters MDR1 and MRP2 in poultry tissues, show an expression pattern in different organs which is rather similar to that of mammalian species, despite the low degree of sequence homology with for example human MDR1 and MRP2. The presented data form a sound basis for forthcoming functional studies. For example, P-gp is not only an essential part of the blood brain barrier, but is also involved in the transmembrane transport of many physiological metabolites such as phospholipids, cytokines, metabolic intermediates and nutrients in mammals (Thiebaut *et al*., 1987; Van Bambeke *et al*., 2003). Moreover, many cytokines are substrates for P-gp, and hence it might play a distinct role in inflammatory reactions (Barnes, 2001). MRP2 provides a major route for the secretion of organic anions, particularly conjugates with glutathione, glucuronic acid. As many of these anions are toxic to the producing or receiving hepatocyte, the efflux of these conjugates is essential for liver function. Inhibition of P-gp, either experimentally or *in vivo* by drugs, toxins or feed components, result in excessive intracellular levels of for example bilirubin glucuronosides, sulfa-lithocholyl-taurine or cysteinyl leukotrienes resulting in hepatocyte damage. In vivo studies in rats and human patients indicate that in individuals with an impaired activity of MRP2 various stages of liver disease occur, mainly due to the inability of the hepatocytes to excrete bilirubin-glucuronides (Konig *et al*., 1999; Homolya *et al*., 2003).

Of major interest in drug development and therapeutics is the fact that various drugs and their conjugated metabolites are substrates for one or more ABC transporter. The knowledge on the tissue specific expression will help to predict the species-specific rate of absorption, distribution and excretion as well as the transfer into vulnerable tissues such as the brain and the reproductive organs (Kusuhara and Sugiyama, 2002; Van Bambeke *et al*., 2003). In turn, drug pharmacokinetics can be altered by compounds (nutrients or other drugs) that modulate the activity of MDR1 or MRP2 in the intestines, kidney, liver and other excretory organs (Cormet-Boyaka *et al*., 1998). However, it should be considered that a number of toxins are substrates of the same transporters (Fardel *et al*., 2005), and hence it needs to be decided at an individual base if the use of modulators of ABC transporters can be used under practical conditions.

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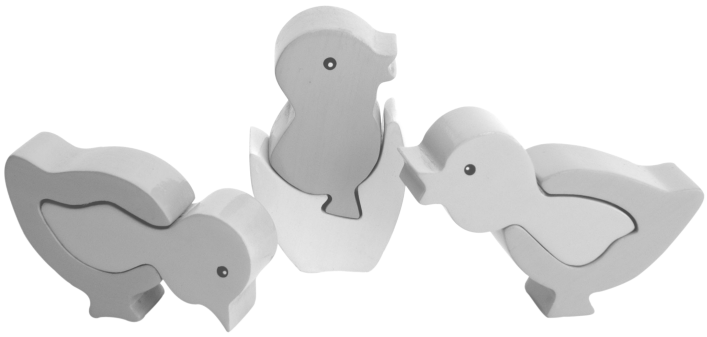
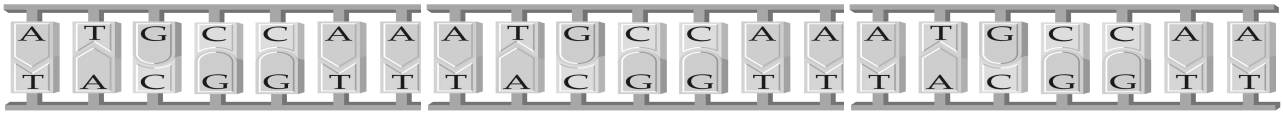
CHAPTER 6





6

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), MRP2 and BCRP are increasingly recognized as determinants of the absorption, tissue distribution and excretion of 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). These cells incorporate the fluorescent dye Rhodamine 123 (Rh-123), and its efflux depends on active transport by ABC transporters. The intracellularly retained Rh-123, as measured by FACS analysis, serves as indicator of transporter activity, which also can be modulated by the use of specific inhibitors. We applied the same model for experiments devoted to the influence of efflux transporters of the transmembrane transport of fluoroquinolones, but replace PBMCs by splenocytes, as these can be easily obtained form smaller animals. Hence, using Rh-123 as a fluorescent dye and cyclosporin A; PSC 833; GF 120918; MK 571 and Ko-134 as typical inhibitors of individual ABC transporters, a functional flow cytometry analysis with splenocytes obtained form healthy chickens was conducted to measure the transport of three fluoroquinolones, commonly used in the therapy of poultry diseases. At these test conditions, the concentration dependent efflux of Rh-123 varied between 59.69%±7.50 at 0.0625 μM Rh-123, and 19.07%±8.31 at 2 μM Rh-123. P-gp inhibitors, such as PSC 833 and GF 120918 significantly inhibit Rh-123 efflux. The selected fluoroquinolones enrofloxacin, danofloxacin mesylate and marbofloxacin did not significantly inhibit P-gp function when tested at concentrations ranging form 1.56 to 50 μM, with exception of the highest concentrations of danofloxacin and danofloxacin mesylate that were found to reduce P-gp activity by approximately 15%. RT-PCR analysis showed that MDR1 mRNA is expressed in chicken splenocytes, but MRP2 and BCRP mRNA is present only at very low levels, explaining the non-significant effects of inhibitors, specific for MRP2 and BCRP. The good reproducibility of the presented results indicates that this method could be used for the screening of other P-gp substrates and modulators in chickens as well.

Introduction

The major membrane transporters involved in the secretion of drugs are P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP). These efflux pumps are members of ATP-Binding Cassette (ABC), a large super-family of transmembrane proteins that use the energy of ATP hydrolysis to transport molecules across cell membranes (Schinkel and Jonker, 2003). ABC transporters are widely distributed in different tissues and their expression has been confirmed also in poultry (Edelmann *et al*., 1999; Barnes, 2001; Haritova *et al*, 2006, see Chapter 5). The activity of these ABC-transporters, however, varies among animal species and even between individuals, as their transcription is linked physiological processes, among others the hormonal status of an animal (Ayrton and Morgan, 2001; Van Bambeke *et al*., 2003; Balayssaca *et al*., 2005; Warrington *et al*., 2004; Albermann *et al*., 2005). In the evaluation of pharmacokinetic properties of 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 oral availability, determine transport across biological barriers, like the blood brains barrier and influence the routes of excretion (Van der Sandt *et al*., 2001; Collett *et al*., 2004; Iida *et al*., 2005; Özvegy-Laczka *et al*., 2005)

Fluoroquinolone antimicrobials have been found to be substrates for one or multiple ABC-drug transporters in mammalian species (Ito *et al*., 1997; Sasabe *et al*., 1997; Naruhashi *et al*., 2003; Sasabe *et al*., 2004) and members of this class of drugs potentially modulate ABC dependent transport. For example, it has been shown that grepafloxacin, levofloxacin and sparfloxacin inhibit the secretion of other substrates across the apical cell-membrane and thus increase the cellular accumulation of the drugs (Cormet-Boyaka *et al*., 1998; Yamaguchi *et al*., 2000, 2001; Lowes and Simmons, 2002; Sikri *et al*., 2004). Therefore, it is assumed that fluoroquinolones administered together with other substrates or inhibitors may modify the pharmacokinetics and thus affect their efficacy. Comparable investigation are lacking in veterinary target animal species, including poultry, where fluoroquinolones are commonly used in the treatment of infectious diseases.

For screening purposes to identify drugs that are substrates for ABC transporters and to monitor potential drug-drug interactions under *in vivo* conditions, a lymphocyte based *ex vivo* model has been widely used (Parasrampuria *et al*., 2001; Laffont, 2002; Steinbach *et al*., 2002; Albermann *et al*., 2005). Peripheral blood mononuclear cells (PBMCs) express P-gp, BCRP and MRP2 albeit at very different levels. 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.

We applied this model using splenocytes from healthy chickens instead of lymphocytes, the latter are difficult to obtain in large numbers from small animals. The effect of various typical inhibitors of individual ABC-transporter was measured, and the data compared with the effect of selected fluoroquinolones commonly used in veterinary

medicine, such as enrofloxacin, danofloxacin mesylate and marbofloxacin. Moreover, in control experiments, we determined the level of expression of multidrug resistance 1 (MDR1), MRP2 and BCRP in the isolated splenoytes by RT-PCR analysis.

Material and methods

Chemicals and drugs

Rhodamine 123 and cyclosporin A were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PSC 833 (SDZ 215-833) was kindly provided by Novartis Pharma AG (Basel, Switzerland). GF 120918 was kindly provided by Glaxo SmithKline (Stevenage, Herts, UK). Ko-143 was kindly provided by Prof. Koomen (University of Amsterdam, the Netherlands). MK 571 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.

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^{o}C) two 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.

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 x *g* at room temperature 22^{o}C. Interphase cells were collected and washed twice with phenol red-free RPMI 1640 complete medium (Gibco, Gibco Grand Island, NY, USA, 32404.014) supplemented with 10% fetal calf serum Gibco (Grand Island, NY, USA) and 1% glutamine. After the second washing step the cell pellets were resuspended in Erylysis buffer containing 1% Bovine Serum Albumin (BSA), 0.155M ammonium chloride, 10mM potassium bicarbonate and 0.1mM disodium EDTA in distilled water, pH 7.2. After 2

minutes, PBS was added and the sample centrifuged at 267 x *g* for five minutes. 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 hemocytometer.

Characterization of lymphocyte-subsets obtained from spleen samples

Cell-samples ($0.3x10^6$ cells) were incubated with the fluorochrome labeled 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 minutes at 4°C in the dark. The cells were subsequently washed twice with FACS buffer, centrifuged for 5 min at 267x *g* and re-suspended in FACS buffer. The cell-associated fluorescence was than measured and quantified by flow cytometry on a Becton–Dickinson FACScalibur fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany).

Measurement of Rhodamine-123 efflux

Splenocytes suspensions were aliquoted into the wells of a 96 well plate ($5x10^4$ cells/well), centrifuged (267 *x g*/5min) 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 hrs. Various concentrations of typical inhibitors and fluoroquinolones were tested. Final concentrations for the typical inhibitors were: cyclosporin A: 4 μM and 20 μM; PSC 833: 1 μM and 5 μM; GF 120918: 0.04 μM and 4 μM; MK 571: 1μM and 25μM, 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 GF 120918 was included as internal 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 Becton–Dickinson 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:

(1) Efflux $_{(t)}$ (%) = 100 · [mFl_0– $mFl_{(t)}$] / mFl_0 ,

$mFl_{(t)}$ and mFl_0 represent the median fluorescence, as the obtained data do not follow a Gausion 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:

(2) Inhibitor (%) = 100 · (1 - AUC Inhibitor / AUC Control).

RNA isolation and cDNA synthesis

Total RNA was isolated using Trizol Reagent (Invitrogen Life Technologies, Cat No 15596-018) according to the manufactures instructions. Briefly, 1.0 ml of Trizol was added to $5x10^6$ splenocytes and homogenized while kept on ice. After centrifugation and incubation for 5 min at 30°C, the supernatant was collected and 0.2 ml chloroform was added and vigorously mixed for 15 s followed by incubation at 30°C for 2 to 3 min. The vials were centrifuged at 12,000 x *g* for 15 min. The aqueous (upper) phase containing the RNA was harvested, and total RNA was precipitated for 10 min at 15-30°C in 0.5 ml isopropanol. After precipitation, the vials were centrifuged at 12,000 x *g* for 10 min. The supernatant was removed and the resulting pellets were washed with 1.0 ml of 75% ethanol. After centrifugation at 7,400 x *g* for 5 min, the supernatant was discarded and the residual ethanol evaporated. The obtained pellet was then re-suspended in RNAse-free water and the RNA concentration was spectrophotometrically quantified at 260 nm and stored at -70°C.

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 20µl. 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.

Real time PCR analysis

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 (Haritova *et al*, 2006, see Chapter 5) 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.

Table 1. Primers used in the PCR. The nucleotide sequences of the PCR primers used to assay gene expression by real-time quantitative PCR.

Gene	Forward primer 5' → 3'	Reverse primer 5' → 3'
MDR1	GCTGTTGTATTTCCTGCTATGG	ACAAACAAGTGGGCTGCTG
MRP2	CTGCAGCAAAATGAGAGGACAATG	CAGAAGCGCAGAGAAGAAGACCAC
BCRP	CCTACTTCCTGGCCTTGATGT	TCGGCCTGCTATAGCTTGAAATC
GAPDH	GTGTGCCAACCCCCAATGTCTCT	GCAGCAGCCTTCACTACCCTCT
Beta-actin	ATGTGGATCAGCAAGCAGGAGTA	TTTATGCGCATTTATGGGTTTTGT

Statistical analysis

The results are presented as mean ± SD. 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.

Results

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 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.

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

All three tested ABC-transporter are expressed in chicken splenocytes, although the levels of expression for BCRP and MRP2 were low in comparison to MDR1 (Fig. 1).

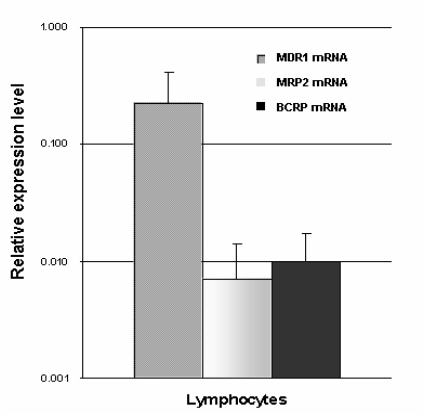

Figure 1. Relative expression levels (mean ± SD) of multidrug resistance 1 (MDR1) mRNA, multidrug resistance-associated protein 2 (MRP2) mRNA and breast cancer resistance protein (BCRP) mRNA in chicken lymphocytes, isolated from spleen and normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin.

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. 2). The fluorescence intensity increased with increasing concentrations of Rh-123 in both subpopulations.

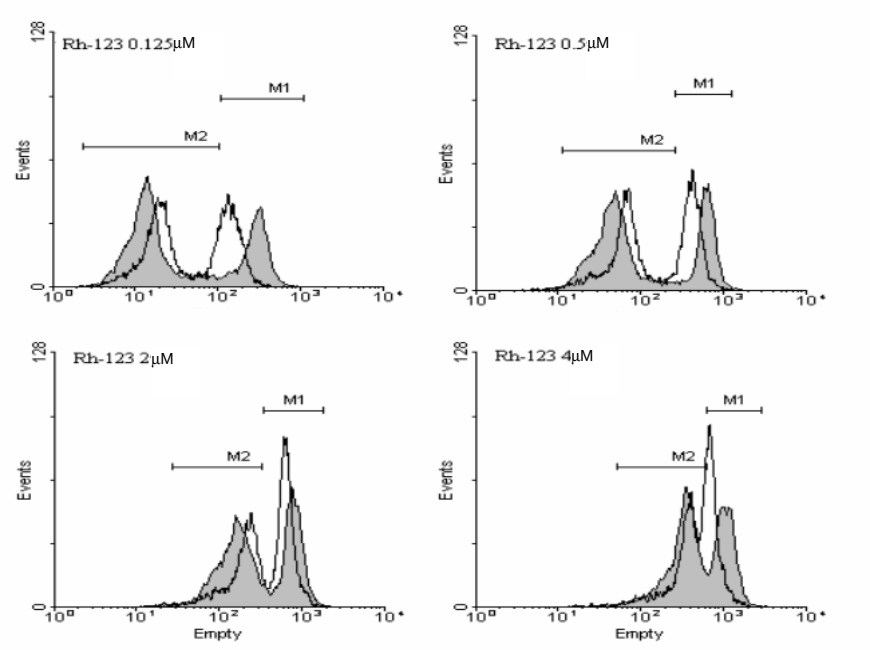

Figure 2. Representative histograms of Rh-123 retention in lymphocytes: two peaks were detected representing two subpopulations of cells (peak M1 and M2). Filled area represents cellular Rh-123 intensity at t=0 h and the unfilled area represents the intensity after 2 hrs of incubation in dye free medium.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

The subpopulation denoted in Figure 2 as M1 exhibited a high percentage of Rh-123 efflux in the two hours following the loading period. In contrast, the subpopulation denoted as M2 in Figure 2 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.

Table 2. Concentration dependent efflux of Rh-123 from lymphocytes (region M1), mean ± SD of 5 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

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, 2 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 between 0.0625 and 0.5 μM) was observed in the presence of GF 120918 (0.4 and 4 μM) and PSC 833 (1 and 5 μM) (P values of <0.001-0.04) (Fig. 3). The most pronounced inhibitory effect was observed when the samples were incubated with 4 μM GF 120918, ranging from 68.13 $\pm$ 6.49 to 84.45 $\pm$ 9.71 % when lymphocytes were incubated with Rh-123 concentrations between 0.0625 and 0.5 μM (P values of <0.003). Cyclosporin A at concentrations of 4 μM significantly inhibits Rh-123 efflux applied at concentration of 0.5 μM (P value of 0.001). The higher concentration of cyclosporine A and investigated concentration of MK 571 did not change significantly the efflux of the fluorescent dye. Excluding the data presented in Table 3, significant inhibition of Rh-123 efflux was not observed in the presence of Ko-134.

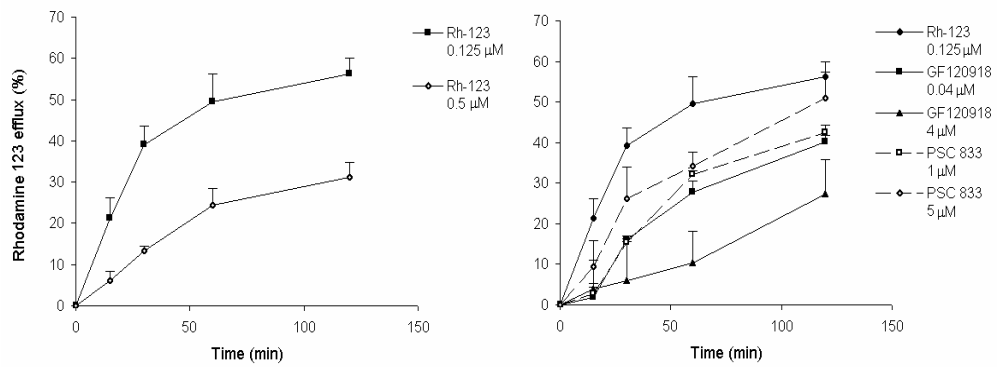

Figure 3. Time-dependent efflux of Rhodamine 123 (Rh-123) in chicken spleenocytes. Efflux was measured over 2 hours of incubation at 41°C. (A) Concentration-dependent efflux of Rh-123 in control samples. (B) Rh-123 efflux in the presence of prototypic inhibitors. Data are presented as mean ± SD from 6 experiments.

Table 3. Inhibition of Rh-123 efflux from splenocytes (subpopulation M1) as affected by inhibitors and individual fluoroquinolones (mean ± SD of 6 experiments).

Concentration (µM)		Inhibition of Rh-123 efflux (%)				
Rh-123	Tested compounds					
		Cyclosporin A	**PSC 833**	**GF 120918**	**MK 571**	**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

*Statisticaly significant difference in comparison to controls at $p<0.008$; # - Statisticaly significant difference in comparison to controls at p=0.02-0.04.

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 and 0.5 μM Rh123 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 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, Fig. 4).

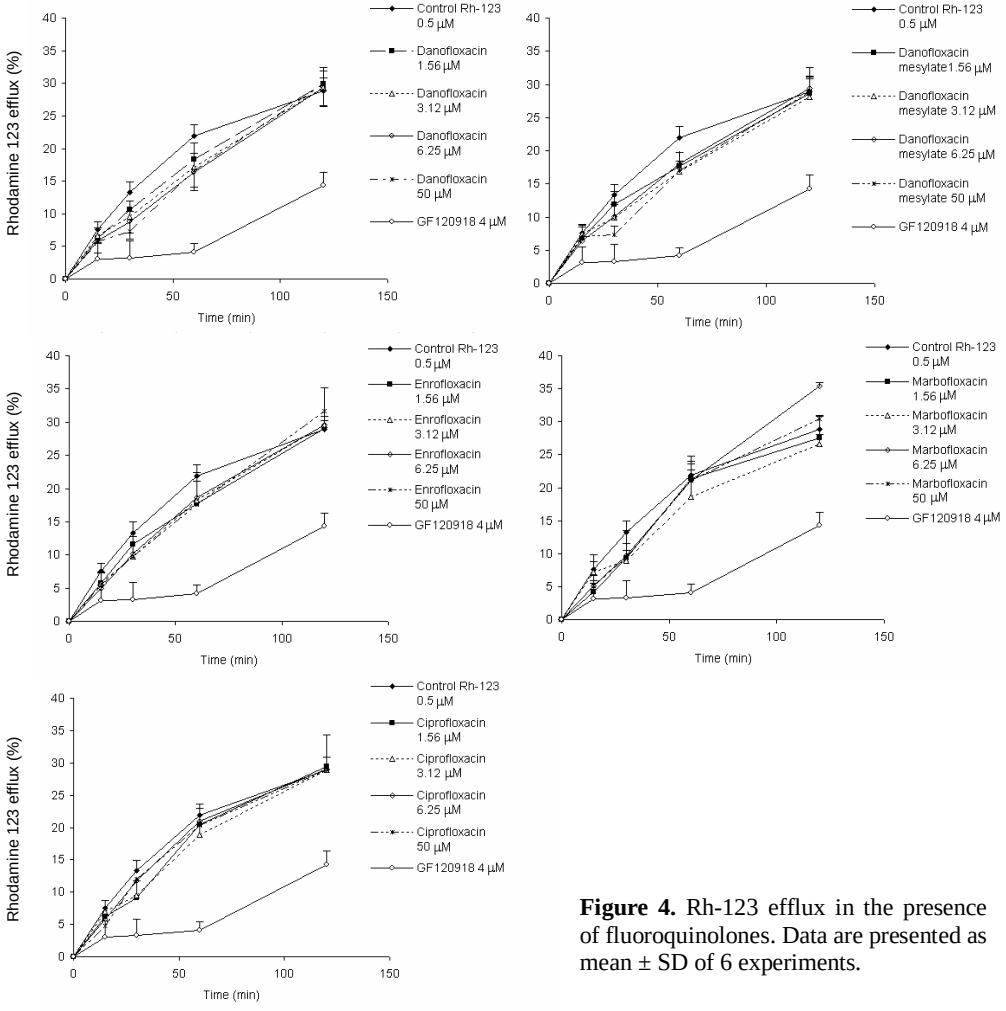

Figure 4. Rh-123 efflux in the presence of fluoroquinolones. Data are presented as mean ± SD of 6 experiments.

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 difference between mammals and chickens in the morphology of blood cells have been described showing that avian thrombocytes may have the same size as a small lymphocytes (Ries *et al.*, 1984; Lacoste-Eleaume *et al.*, 1994; Luhtala *et al.*, 1997; Luhtala, 1998). 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 (Bertram *et al.*, 1998; Kim *et al.*, 2003). This fact jeopardizes the use of lymphocytes as an easy model for transport experiments, and hence we used splenocytes form healthy donor animals as surrogates for peripheral blood mononuclear cells, as previous experiments had demonstated that these splenocyte fraction contain at maximum 5% thrombocytes (Lacoste-Eleaume *et al.*, 1994; Bertram *et al.*, 1998; Kim *et al.*, 2003). 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 (Drach *et al.*, 1992; Beck *et al.*, 1996; Steinbach *et al.*, 2002).

The function of efflux transporters in isolated splenocytes was estimated by the rate of efflux of the fluorescent dye Rh-123 from cells first loaded with Rh-123 and than 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 rate differed (Witkowski *et al.*, 1993).

Lymphocytes express predominantly P-gp and we confirmed this by RT-PCR analysis of the used splenocyte cultures. These data indicated the expected enough high expression of MDR1, the gene encoding for P-gp. In addition small amounts of MRP2 and BCRP mRNA were detected. The hypothesis was confirmed when we measured the effect transporter-specific inhibitors and found a significant effect only for GF 12918, whereas the other inhibitors, including cyclosporin A, MK571, PS C833 and Ko-143 exerted only a

very week inhibitory effect on the Rh-123 efflux. Also Maliepaard *et al.* (2001) found no significant staining for BCRP in blood cells, i.e., erythrocytes, leukocytes, and platelets in humans. Nevertheless, several studies had suggested previously that *ex vivo* lymphocyte models could be used to study functional activity of other ABC transport proteins in mammals as well (Parasrampuria *et al.*, 2001; Steinbach *et al.*, 2002; Albermann *et al.*, 2005), but at present extensive data are only available for P-gp (Eisenbraun *et al.*, 2000).

The use of Rh-123 in flow cytometry as a probe for P-gp activity is highly sensitive, although it can not entirely be excluded that other proteins, such as MRP1 contribute to its transport (Taniguchi *et al.*, 1996; Webb *et al.*, 1996; Steiner *et al.*, 1998; Daoud *et al.*, 2000). In chickens, P-gp mediated efflux of Rhodamine 6G was found to be inhibited by PSC 833 (Edelmann *et al.*, 1999), however, to the best of our knowledge no other studies have addressed the function of P-gp and related transporters in poultry as yet.

In humans, also BCRP seem to contribute to the transport of Rh-123 (Doyle *et al.*, 1998). Comparing the obtained data from poultry splenocytes with findings from other animal species (Laffont, 2002), it must be concluded that chicken lymphocytes express a limited amount of P-gp activity, which is readily saturable as demonstrated by the non-linearity of Rh-123 uptake at higher concentrations including those (4 µM) found to be optimal for transport studies in lymphocytes from mammalian species.

As it could not ruled out the splenocytes used for *in vitro* experiments also express other transporters than P-gp, we used a panel of known inhibiters to further characterize Rh-123 efflux. Cyclosporin A is a broad-spectrum MDR substrate and modulator of the activity of P-gp, MRP2 and BCRP (Huet *et al.*, 1998; Egashira *et al.*, 1999; Hesselink *et al.*, 2005; Qadir *et al.*, 2005; Tsujimura *et al.*, 2005). A significant inhibition of the Rh-123 efflux in chicken splenocytes by cyclosporin A was only observed at a concentration of 4 µM, whereas higher concentration of cyclosporin A (20 µM) did not affect Rh-123 efflux, suggesting both inhibition and stimulation of efflux (Loor *et al.*, 2002; Tarasova *et al.*, 2005). GF 120918 and PSC 833 are potent inhibitors of P-gp function as well, with a high specificity of PSC 833 for P-gp, while GF 120918 is also an inhibitor of human BCRP albeit with lower potency (Mayer *et al.*, 1997; Lin, 2003; Wang *et al.*, 2003). In consideration of these previous findings it can be suggested that the inhibition of Rh-123 efflux by PSC 833 and GF 120918 at a concentration of 0.4 µM are mainly attributable to the inhibition of P-gp. When GF 120918 was applied at a concentration of 4 µM, a more pronounced inhibition was observed as compared to PSC 833 and the lower concentrations of GF 120918. This finding suggested an additional role for BCRP. Therefore we tested also Ko-143 as a highly potent inhibitor of BCRP (de Bruin *et al.*, 1999), and with affinity for P-gp (Raaijmakers *et al.*, 2005). Ko-143 applied at the concentrations 0.04 and 1.0 µM decreased Rh-123 efflux to a similar extend, however, at a lower level than was observed for cyclosporine A, PSC 833 and GF 120918. Although the measured inhibition was not dose-dependent, these data suggest a role for BCRP in Rh-123 efflux, albeit at a lower level than P-gp. MK 571 inhibited Rh-123 efflux in the splenocytes from chicken as well. MK 571 is a putative inhibitor for MRP's, inclusive MRP2 (Lowes and Simmons, 2002), 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 P-gp and to a lesser extend by BCRP, while the role of a MRP2 remains to be elucidated.

Since the concentrations of >1 µM Rh-123 resulted in highly variable results indicative for a saturation of the transporter capacity, the lower concentrations of 0.5 µM and 0.125 µM Rh-123 as a loading concentrations were used in all experiments with fluoroquinolones.

A significant inhibition of Rh-123 efflux was only observed with danofloxacin and danofloxacin mesylate. However, 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 fluoroquinoles 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. PSC 833 and GF 120918 interacted with this inhibition hence pointing towards a prominent role of P-gp in this process. All other tested drugs (enrofloxacin sulfate, marbofloxacin and ciprofloxacin) only tentatively inhibited Rh-123 efflux. These findings suggest that fluoroquinolones are in general weak substrates for efflux transporters and do not inhibit this transporters to a significant degree. Hence the risk for undesirable drug-drug interactions and increased side effects, in cases in which multiple therapies are required, seem to be low. In turn, the finding that some flouroquinolones are substrates for P-gp contributes to the understanding of 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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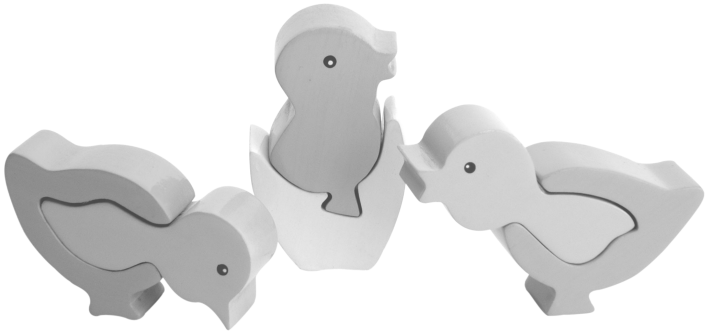
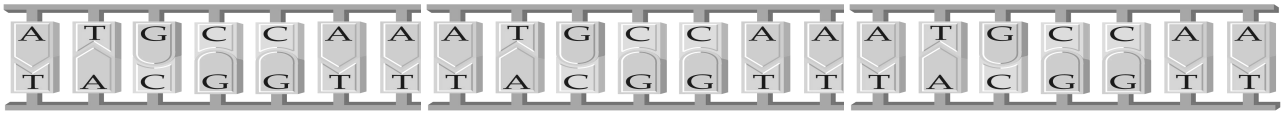
CHAPTER 7





7

Effects of the Fluoroquinolone Danofloxacin on the Expression of MDR1, MRP2 and BCRP mRNA in Tissues of Healthy Turkeys

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Abstract

MDR1, MRP2 and BCRP are members of the superfamily of ABC membrane transporters that export a large variety of structurally diverse substances out of cells hence being an integral part of various biological barriers. The expression of ABC transporters shows specific-differences, as evidenced from the results obtained from laboratory animal species as well as human. Here we report for the first time the tissue distribution of the ABC transporters MDR1, MRP2 and BCRP in turkeys. Tissue samples of male and female BUT9 turkeys dissected from crop, proventriculus, duodenum, proximal and distal jejunum, ileum, caecum, colon, liver, kidney, lung, brain, adrenal glands, ovaries and testis were collected for quantitative RT-PCR analysis, for which a set of primers was designed. The mRNA levels of MDR1, MRP2 and BCRP were normalized against GAPDH and beta-actin. Secondly, in consideration of previous findings suggesting that fluoroquinolones are substrates of ABC transporters, and might modulate their expression, the effect of two oral dose regimes of danofloxacin mesylate, a representative of the group of fluoroquinolones licensed for the use in veterinary medicine, on the levels of MDR1, MRP2 and BCRP mRNA expression was investigated. MDR1 and BCRP mRNA expression was detected in all tissue samples, and the highest levels were measured in the small intestines (duodenum and jejunum), liver and kidney. Expression levels did not significantly vary between individual animals or between sexes. In contrast, the tissue distribution of MRP2 mRNA was less constant and some tissues seem to lack any significant expression. Danofloxacin treatment resulted in slight up-regulation of the measured transporters at the transcriptional level. These changes were not significant and hence did not affect the pharmacokinetic parameters of danofloxacin mesylate, as demonstrated by the control of principle pharmacokinetic parameters.

Introduction

MDR1, MRP2 and BCRP are membrane proteins belonging to the family of ABC efflux transporters. They facilitate the transport of numerous substrates out of the cell using ATP hydrolysis as energy source. These efflux transporters form an integral part of the intestinal barrier, blood-brain barrier, blood-testes barrier and the placental syncytiotrophoblasts and are present at apical excretory sites in the liver, the kidneys, the lung and adrenal gland in mammals (Trezise *et al*., 1992; Schinkel *et al.*, 1994; Cherrington *et al*., 2002; Mouly and Paine, 2003). In turn, can limit the intestinal uptake of orally administered drugs, facilitate their hepatobiliary excretion, and restrict their distribution (Thiebaut *et al.*, 1987; Alleen and Schinkel, 2002; Lin and Yamazaki, 2003). Species differences in the level of expression, as well as induction or inhibition of these transporters by food/feed components can contribute to inter-individual differences in drug disposition (Soldner *et al*., 1999; Goh *et al.*, 2002; Pietro *et al*., 2002). Moreover, drug–drug interactions have been described to occur (Greiner *et al*., 1999; Dresser *et al*., 2003). Initially ABC transporters were recognised as multi-drug resistance genes, when in the course of a repetitive use of cytostatic agents in the treatment of cancers, not only rate of absorption after oral application but also the efficacy decreased (reviewed by Leonard *et al*., 2003). A typical example is tamoxifen that increases its own excretion into the rat bile canaliculus, an effect which is most likely mediated through increased MDR1b gene expression (Riley *et al*., 2000). Moreover, other structurally unrelated drugs such as propranolol, verapamil and rifampicin induced rapidly MDR1 mRNA and P-gp protein expression four to six-fold (Collett *et al.*, 2004). Finally, results form Zhao *et al*. (2002a) showed that the fluoroquinolone drug sparfloxacin has a strong inhibitory effect on P-gp, which was more pronounced than that of other substances of the same group, i.e. norfloxacin, enoxacin, ofloxacin, lomefloxacin and grepafloxacin.

While the expression of ABC transporters has been extensively investigated in rodents as well as in human tissues, little information is available about the expression in other animal species. Data in farm animals are scarce. In chickens, few data are available about the MDR1 gene product, P-gp. Edelmann *et al.* (1999) and Barnes (2001) detected P-gp in liver, duodenal, jejunal, ileal, kidney, caecal, lung, brain and ocular tissues, but not in the adrenal gland. Investigation of chicken tissues indicated the expression of MRP2 mRNA in the small intestines, liver, kidney and lung (Haritova *et al*., 2006; see Chapter 5).

Here we describe the tissue distribution of MDR1, MRP2 and BCRP in healthy turkeys, as analysed by quantitative RT-PCR analysis (qPCR). Tissue samples investigated were crop, proventriculus, duodenum, proximal and distal jejunum, ileum, caecum, colon, liver, kidney, lung, brain, adrenal glands, ovaries and testis. The mRNA levels of MDR1, MRP2 and BCRP were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin. In addition, the influence of single and repetitive oral administration of danofloxacin mesylate, a representative of the group of fluoroquinolones

licensed for the use in veterinary medicine on the levels of MDR1, MRP2 and BCRP mRNA expression was investigated.

Materials and Methods

Animals

Twelve turkeys (breed BUT 9), 8-weeks old from both sexes were obtained from an experimental poultry farm in Stara Zagora, Institute of Animal Husbandry and included in the two experiments. The body weight of the male turkeys varied between 1.9 - 2.6 kg and that of the females between 1.8 - 2.4 kg. The animals were kept at 25^{o}C. All animals had free access to water and standard commercial feed, without additives. Animals were sacrificed and the given time points and tissue samples were immediately dissected, snap-frozen in liquid nitrogen and stored at –70^{o}C until analysis.

Studies with Danofloxacin

Danofloxacin mesylate (Advocin 180, Pfizer, NL 9945 UDA, Part. No 2058806, V0704, 18 % sterile solution) was used for oral treatment as 0.9% w/v solution, diluted with sterile water. Two studies were conducted with different treatment regimes:

First experiment.

Four animals (three female and one male) were treated individually (crop tube) with danofloxacin at a dose rate of 6 mg/kg bw for 5 consecutive days, S.I.D.. Two additional animals (one female and one male) served as controls and remained untreated. Blood samples (0.8 ml) were collected form the *V. brachialis* prior to treatment and at 3, 6, 9, 12, 24, 99, 102, 105, 108 and 120 hours after the first drug administration. Serum was collected after centrifugation at 1800 x *g* for 15 min and stored at -25^{o}C prior to analysis. At the end of sampling period, all animals were euthanized and tissue samples from crop, proventriculus, duodenum, proximal and distal jejunum, ileum, caecum, colon, liver, kidney, lung, brain, adrenal gland, ovaries, oviduct and testes were immediately collected and snap-frozen in liquid nitrogen. Samples were stored at -70°C until analysis.

Second experiment.

Four animals (two female and two male) were treated individually with danofloxacin at a dose rate of 6 mg/kg bw for 5 consecutive days as in experiment A and serum samples were taken at the same time intervals (up to 24 hours after the first drug administration) as mentioned above. Thereafter animals remained untreated for an interim period of 5 days. At day 11, all animals received an additional dose of danofloxacin (6 mg/kg bw) and serum sampling was repeated at 3, 6, 9, 12 and 24 hours after this drug administration. One female and one male animal remained untreated and served as controls. After the last blood sampling all animals were euthanized and tissue samples from the same tissues as in first experiment were collected and stored under similar conditions.

Determination of danofloxacin in serum and tissues

The serum concentrations of danofloxacin were determined applying an HPLC method as described by Garcia *et al.* (2000) with minor modifications. Briefly, serum samples (100 µl) were diluted with 400 µl of 0.1M phosphate buffer pH 7.4 and vortexed for 0.5 min. After adding 3 ml dichloro-methane, the samples were mixed again for 1 min and centrifuged for 6 min at 1000 *x* g, at 4°C. The organic phase was collected and evaporated in a vacuum evaporator at 40°C. The residue was dissolved in 100 µl of demineralised water. A 20 µl aliquot was injected into an HPLC system comprising a Spherisorb ODS-2-250x4.6 mm 5µM column, a high pressure pump (Model 300) and fluorescence detector (Jasco, Model 821 FP) and an autoinjector (Gyna 50). The area under curve was integrated by Chromeleon software (Separations, H.I. Ambacht, The Netherlands). Excitation and emission wavelengths were set at 280 nm and 440 nm, respectively. The mobile phase consisted of acetonitrile in aqueous solution (20:80, v/v) of potassium dihydrogenphosphate (0.02 M) and tetrabutylammonium hydrogenphosphate (0.02 M) in water. The pH was adjusted to 3.0 with phosphoric acid (85%). The flow rate was 1.0 ml/min. The concentration of danofloxacin was calculated by comparing the peak area of a standard curve derived from spiked serum sample with the peak area of the individual samples. The limit of quantification was 0.05 µg/ml.

The tissue samples (0.5 g) were homogenized in 0.5 ml of phosphate buffer with an Ultraturax at high speed for a few seconds. To this homogenate, 6 ml of dichloromethane were added, and extraction and analysis completed as mentioned above for the serum samples. Pilot experiments with spiked tissue samples demonstrated that the overall recovery rate in tissue specimen exceeds >73%.

Standard dilutions of danofloxacin mesylate were prepared in serum obtained from untreated turkeys at concentrations of 1.0, 0.75, 0.50, 0.25, 0.10, 0.05 and 0.01 µg danofloxacin per ml and subjected to HPLC analysis as well. Linearity of the standard curve was confirmed by the test for lack of fit (non significant, p=0.274) and value of *r* was 0.994. The intra-assay and the inter-assay coefficients of variation (CV) were calculated to be 2.27 and 10.68, respectively.

Pharmacokinetic analysis

Pharmacokinetic analysis of data was performed using a non-compartmental analysis (WinNonlin 4.0.1., Pharsight Corporation, 800 West El Camino Real, Mountain View, CA, USA). The area under the serum-concentration-time curve (AUC) was calculated by the method of trapezoids with extrapolation to infinity. The calculated parameters were used to simulate serum concentrations up to 120 h after a daily repeated administration for five days (Gibaldi and Perrier, 1982). The simulation step was 0.5 hours.

RNA isolation

Total RNA was isolated using Trizol Reagent (Invitrogen life technologies, Cat No 15596-018). Briefly, between 50 to 90 mg of tissue was added to 1.0 ml of Trizol, placed in

sterile polypropylene vials, kept on ice, and mechanically homogenized (Ultraturax). After centrifugation and incubation for 5 min at room temperature, 0.2 ml chloroform was added to the supernatant and the mixture homogenized. The vials were then centrifuged at 12,000 *x* g for 15 min. The aqueous (upper) phase containing the RNA was harvested, and total RNA was precipitated for 10 min with 0.5 ml isopropanol. After precipitation, the vials were centrifuged again at 12,000 *x* g for 10 min. The supernatant was removed and the resulting pellets were washed with 1.0 ml of 75% ethanol and centrifuged again at 7,400 *x* g for 5 min. After centrifugation, the supernatant was discarded and the pellet was allowed to dry to minimize residual ethanol. The obtained pellet was re-suspended in Rnase-free water. The RNA was quantified spectrophotometrically at 260 nm (ND-1000, Nanodrop technologies). Samples were stored (< 7 days) at -70^{o}C prior to analysis.

cDNA synthesis

First strand cDNA's were synthesized from 1 µg total RNA with the iScript^{tm}cDNA Synthesis Kit (***Bio-Rad*** Laboratories, USA), containing both oligo (dT) and random hexamer primers. In brief, to a master mixture (prepared ***ex tempore***), containing 5x iScript Reaction Mix and iScript reverse transcriptase, 1 µg of total RNA in sterile nuclease free water was added to give a final volume of 20 µl. This reaction mixture was incubated for 5 min at 25^{o}C, and at 42^{o}C for 45 min followed by heat inactivation of the enzymes at 85^{o}C for 5 min and a final fast-cool step. The obtained cDNA was diluted 1:10 with sterile Rnase-free water and stored at -20^{o}C until use.

Sequence analysis

Primers were developed complementary to chicken P-gp (NCBI accession number NM_204894), human ABCC2 (NCBI accession number NM_000392) and the predicted sequence for chicken ABCG2 (XM_421638) and were commercially produced (Isogen, the Netherlands). The primers that were used for sequence analysis are presented in Table 1. Pooled cDNA from liver, kidney, jejunum and adrenal gland was amplified in a PCR reaction containing 2.5 U platinum Taq polymerase, 0.8 pmol/µl of each primer, 200µM dNTP's and 1.5 mM $MgCl_2$ (Invitrogen, California, USA). The cDNA was amplified with a denaturation step at 94^{o}C for 1 min, an annealing step at a temperature gradient for 30 sec and an extension step at 72^{o}C for 1 min. The PCR-products were analysed by gel-electrophoresis and visualized by ethidium bromide staining. Specific PCR products were used for sequence analysis.

The ABI PRISM Big Dye Terminator v3.0 ready reaction cycle sequencing kit (Applied Biosystems) with fluorochrome labelled dideoxynucleotides was used for the preparation of terminated DNA chains in a thermal cycler according to the manufactures instructions, and subsequently purified using Sephadex G-50. The DNA sequences were than analysed in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences were assembled using the SeqMan software from DNA STAR (Madison, USA).

Table 1. Primers used for sequence analysis of turkey ABC transporters.

Gene		Location	Forward primer 5' ® 3'	Reverse primer 5'® 3'
MDR1	NM_204894	1500-2466	GGATCTGAAGAGCCTGAATGTAAG	GCCAAATGCAAAACCCTGAA
		2168-3011	AAGCATGAAAAAGCCAGGAGAACC	CCAAAGCGGAAACAGCCAGCATA
		2737-3205	GCAGTGGCAGGAATGATTGAAATG	CATCCTCGCGGTAACTGTCTATTGG
		3137-3809	AGATATCAGCAGCCCACTTGTTTG	GTGCGACCTTCTCTGGCTTTATC
MRP2	NM_000392	3496-4489	TGTCACCAGGTCCCCAATCTAC	GGCGAACTCGTTTTGGATGGT
BCRP	XM_421638	1450-2064	TCCAAGAAGGGGCACGAGATTA	AAGCCAGCCCATTACAGAAGG
Beta-actin	NM_205518	28-464	CGCCCCGGCTCTGACTG	CATGGCTGGGGTGTTGAA
		217-685	AAGACAGCTACGTTGGTGATGAAG	CTCTCGGCTGTGGTGGTGA
		397-885	TGAACCCCAAAGCCAACAGA	CACAGGACTCCATACCCAAGAAAG
		653-1118	GAGAGGCTACAGCTTCACCACCAC	GGACAGGGAGGCCAGGATAGAG
		846-1258	GCCTCTTCCAGCCATCTTTCTT	TTTTATGCGCATTTATGGGTTTTG

MDR1 - multidrug resistance 1; MRP2 - multidrug resistance-associated protein 2; BCRP – breast cancer resistance protein; NCBI – The National Center for Biotechnology Information.

qPCR analysis

Primers complementary to turkey MDR1, MRP2 and BCRP and the internal controls, GAPDH and beta-actin cDNA's were designed and commercially synthesized (Isogen Bioscience BV, The Netherlands). The primers used (Table 2) were selected based on specificity and efficiency by qPCR analysis of a dilutions series of pooled cDNA at a temperature gradient for primer-annealing and subsequent melting curve analysis, agarose gel-electrophoresis and nucleotide sequence analysis (data not shown). The reaction mixture for the qPCR containing 10 µl of the diluted cDNA, was mixed with 15 µl iQTM SYBR Green Supermix (Bio Rad Laboratories Inc., USA), forward and reverse primers (final concentration of 0.4 pmol/µl for each primer) and sterile water according to the manufactures instructions. Q-PCR was performed using the MyIQ single-colour real-time PCR detection system (Bio-rad, Hercules, CA) and MyiQ System Software Version 1.0.410 (Bio Rad Laboratories Inc., USA). A standard dilution series of pooled cDNA including a blanco control was run in the same plate as the samples.

Table 2. Nucleotide sequences of the primers used for real-time quantitative PCR analysis.

Gene	NCBI accession number	Forward primer 5' ® 3'	Reverse primer 5' ® 3'	Tm (^{o}C)
MDR1	AY859758	CATTGGCCCTTGTTGGTAGC	TTCAGTGTTTTTGCATCTTTGTCA	58.1
MRP2	AY856867	TCCTGCAGCAAAATGAGAAGACGA	AAGACCACCAGGCTCCCAACAAAC	65.0
BCRP	Not given yet	CCTACTTCCTGGCCTTGATGT	TCTGCCTGCTATAGCTTCAAACC	63.1
GAPDH	U94327	ATGTGCCAACCCCCAATGTCTC	AGCAGCAGCCTTCACTACCCTCTT	64.5
Beta-actin	AY942620	ATGTGGATCAGCAAGCAGGAGTA	TTTATGCGCATTTATGGGTTTTGT	64.0

MDR1 - multidrug resistance 1; MRP2 - multidrug resistance-associated protein 2; BCRP – breast cancer resistance protein; GAPDH - glyceraldehyde 3-phosphate dehydrogenase; NCBI – The National Center for Biotechnology Information; Tm – optimal annealing temperature.

Data analysis

Analysis of the target genes as well as the internal control genes followed the algorithms described by Radonic *et al.* (2004). Gene expression was quantified using algorithms outlined by Vandesompele *et al.* (2002) and from the geNorm manual, available on the web site http://medgen.ugent.be/~jvdesomp/genorm/.

Statistical analysis

The results are presented as mean ± SD. All data were analysed with the Statistica 6.1 computer program (Statistica for Windows, StatSoft, Inc., USA, 1984-2002). Statistical analysis was done according to Friedman-ANOVA, Wilcoxon post-hoc test (for samples derived from the same animal) and the Mann-Whitney post-hoc test (for the comparison of samples derived from different animals).

Results

Levels of expression of P-gp, MRP2 and BRCP in non-treated healthy animals

We have initially analysed the partial sequences of P-gp, MRP2, BCRP, GAPDH and beta-actin from turkey and found a high homology with the chicken. Homology with chicken MDR1 was 95.3%, with MRP2 and BCRP were 94.8% and 92.5%, and with beta-actin and GAPDH were 98.7% and 97.7%, respectively. These sequences were deposited to and are publicly available now at the NCBI website as follow: MDR1 (AY859758), MRP2 (AY856867), beta-actin (AY942620) and GAPDH (U94327).

All tissues expressed measurable concentrations of GAPDH- and beta-actin mRNA, with similar C_t values. The relative levels of expression of MDR1, MRP2 and BCRP mRNA in different tissues from turkeys are depicted in Figure 1. MDR1 mRNA was detected in all tested tissues. Comparing different parts of the intestinal tract, very low levels were found in the crop and the proventriculus, whereas the relative level of MDR1 mRNA expression was almost equal in duodenum and jejunum, and slightly lower in ileum. After a further decline in the caeca, MDR1 mRNA levels were higher again in the colon. Comparable levels were found also in the liver, kidney and brain. Low levels were found in the lungs and the adrenal gland. MDR1 mRNA was found also in immature testes and ovaries (data not shown). For MRP2, very low but still detectable levels were found in the crop, proventriculus and in the caeca. Low and rather similar MRP2 mRNA levels were found in ileum, colon, lung, brain and adrenal gland. High levels were detected in the duodenum and jejunum as well as in the liver and kidney. In immature testes and ovaries of the young males and females, respectively, low levels of MRP2 mRNA were detected (data not shown).

BCRP mRNA was found as well in all tested tissues. In the gastrointestinal tract, the lowest levels were obtained in the crop, but they were apparently higher than those of MDR1 and MRP2 mRNA. The highest amounts were found in the small intestines (duodenum, jejunum and ileum) and liver, and medium levels in the proventriculus, colon

and caeca. Lungs, kidneys and adrenal glands show medium levels of BCRP mRNA, but again these levels appeared higher than those of MDR1 and MRP2. Medium were levels of BCRP mRNA in immature testes, ovaries and the oviduct.

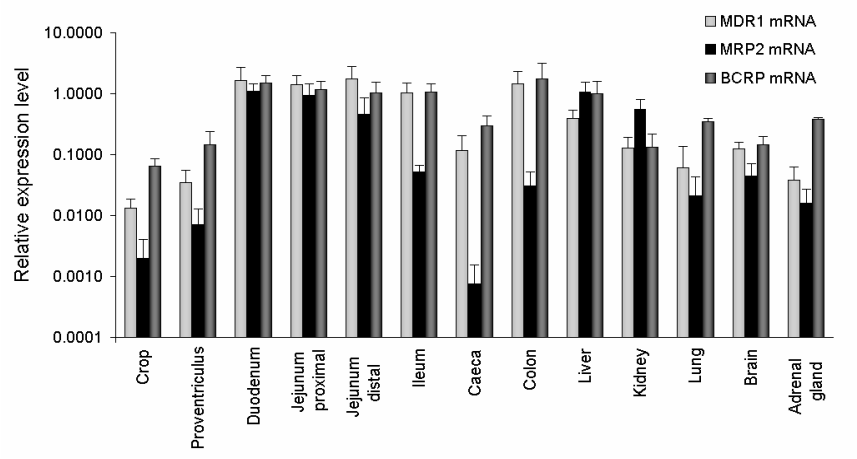

Figure 1. Relative expression levels (mean ± SD) of multidrug resistance 1 (MDR1) mRNA, multidrug resistance-associated protein 2 (MRP2) mRNA and breast cancer resistance protein (BCRP) mRNA in turkey tissues, normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin.

Relative level of expression of MDR1, MRP and BCRP mRNA in turkey tissues after treatment with danofloxacin mesylate.

The used internal control genes (housekeeping genes) remained nearly unaffected by the treatment, but after both experimental periods, changes in a similar direction (up- or down-regulation as described) were found in mRNA levels of beta-actin and GAPDH in the colon, ileum and lung tissues. Beta-actin mRNA levels were also altered in the adrenal gland and jejunum, and to a lesser extend in the kidney. In the other investigated tissues there were no changes. This variability indicates again, that expression levels of individual genes can not be analysed by statistical matters for significant differences.

MDR1 mRNA: After the first experimental period (5 days oral treatment with danofloxacin mesylate), a moderate increase in the expression of MDR1 was found in all tissues (Fig. 2). In lung and colon the house-keeping genes were up-regulated as well, and hence the increase of relative levels of MDR1 in these tissues might be underestimated. The opposite tendency was observed in the samples of the ileum; here the house-keeping genes were down-regulated (albeit to a lesser degree than in the lung and colon). After the second experimental period (5 days in treatment with danofloxacin mesylate; 5 days without treatment and one treatment at day 11) there were apparently no persistent changes in the

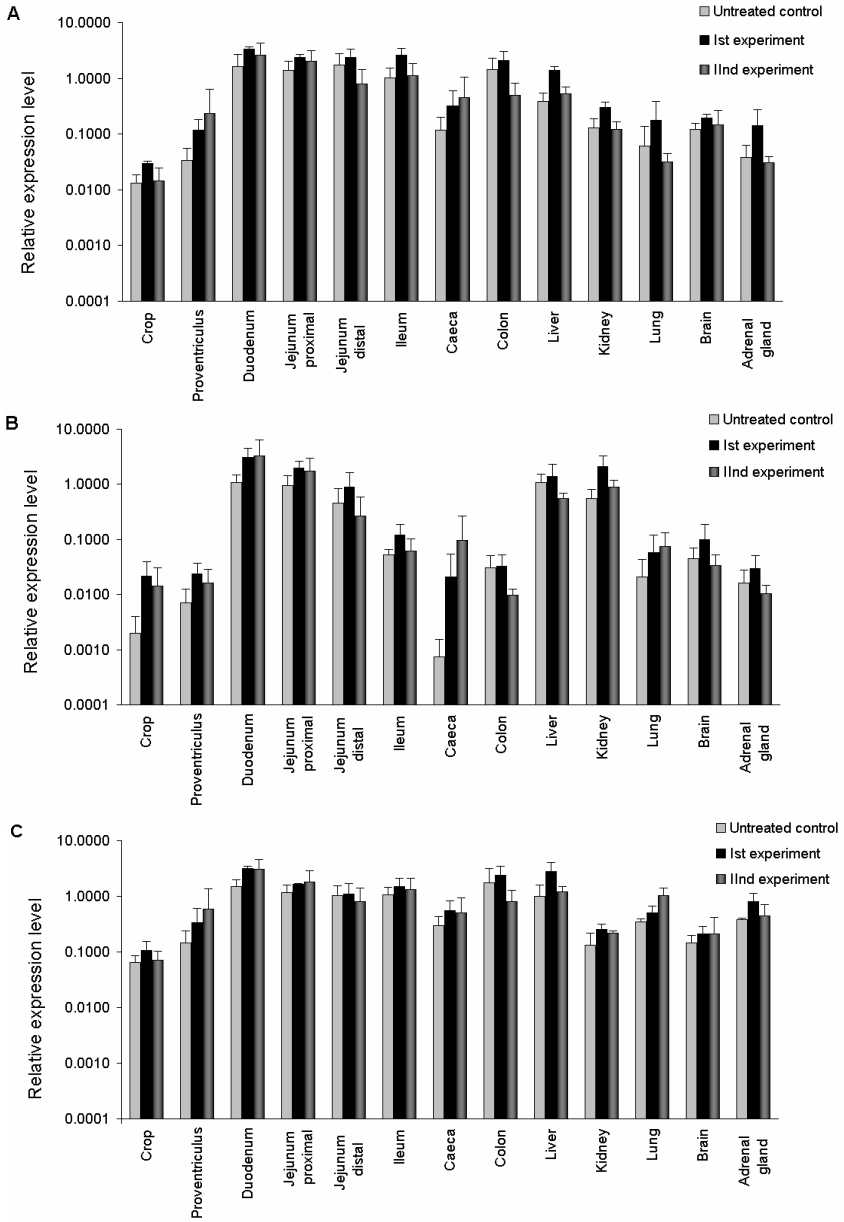

Figure 2. Relative levels of expression (mean ± SD) of multidrug resistance 1 (MDR1) mRNA (A), multidrug resistance-associated protein 2 (MRP2) mRNA (B) and breast cancer resistance protein (BCRP) mRNA (C), in tissues of untreated turkeys (n=4); in birds orally treated with danofloxacin mesylate at a dose rate of 6 mg/kg bw for 5 consecutive days (Ist experiment, n=4) and in animals orally treated with danofloxacin mesylate at a dose rate of 6 mg/kg bw for 5 consecutive days, followed by a therapy-free interval of 5 days, and a single treatment on the 11th day, before being sacrificed 24 hrs later (n=4).

level of expression and no significant up-regulation of MDR1. The slight decrease in the expression levels in the colon and lung could be associated with the up-regulation of GAPDH and beta-actin mRNAs.

MRP2 mRNA: After the 5-days treatment with danofloxacin mesylate (first experiment) an induction of MRP2 at transcriptional level was found in all investigated tissues. In the second experiment a slight increase in the levels of MRP2 mRNA was detected in the most of the investigated tissues. mRNA of this gene of interest shows a tendency towards down-regulation in the distal jejunum, colon, liver, brain and adrenal glands.

BCRP mRNA: In both experiments a tendency towards slightly increased levels in BCRP mRNA were seen, but none of these alterations were significant (Fig. 2).

Pharmacokinetic analysis.

Serum concentrations and pharmacokinetic parameters are presented in Fig. 3 and Table 3. There were no statistically significant differences in the pharmacokinetic parameters between the two treatment groups.

Tissue levels

Twenty four hours after the 5 days treatment tissue concentrations were highest in kidney (0.48±0.04 μg/g), duodenum (0.45±0.17 μg/g), jejunum (0.38±0.21μg/g) and liver (0.36±0.07 μg/g). Lower concentrations were measured in caeca (0.28±0.00 μg/g), ileum (0.12±0.08 μg/g) and colon (0.15±0.05 μg/g), and very low concentrations in the lung (0.07±0.02 μg/g).

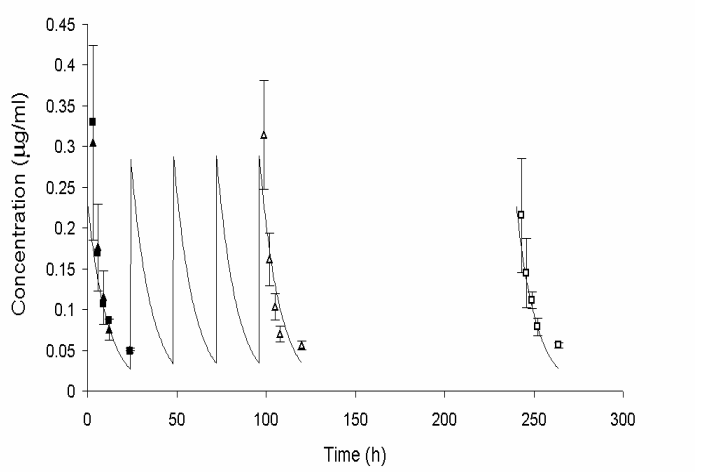

Figure 3. Serum concentrations (mean ± SD) of danofloxacin mesylate given orally at a dose of 6 mg/kg to turkeys for 5 consecutive days (1st experiment; ▲ - after the first dose and Δ - after the last dose; n=4) and during the 12 days experiment (2nd experiment; ■ - after the first dose and □ - after the last dose; n=4). Data in between are simulated.

Table 3. Pharmacokinetic parameters of danofloxacin mesylate at a dosage of 6 mg/kg after oral administration to turkeys (data represent mean ± SD of 4 individual animals).

Pharmacokinetic parameters (units)	1st experiment - 5 days		2nd experiment – 12 days	
	After the first dose	After the last dose	After the first dose	After the last dose
non-compartmental analysis				
β (h^{-1})	0.09 ±0.02	0.07 ±0.01	0.09 ±0.01	0.07 ±0.04
$t_{1/2\beta}$ (h)	7.76 ±1.65	9.77 ±1.91	7.56 ±0.76	11.42 ±5.21
MRT (h)	12.68 ±2.49	15.84 ±3.07	12.34 ±0.74	18.16 ±7.63
C_{max} (µg/ml)	0.31 ±0.12	0.31 ±0.07	0.33 ±0.06	0.22 ±0.07
$AUC_{3\text{-}24h}$ (µg.h.ml^{-1})	2.49 ±0.98	2.60 ±0.26	2.54 ±0.73	2.14 ±0.36
$AUC_{0\text{-}\infty h}$ (µg.h.ml^{-1})	3.08 ±0.98	3.40 ±0.12	3.16 ±0.60	3.11 ±0.51

β - elimination rate constant; $t_{1/2\beta}$ - terminal elimination half-life; C_{max} - maximum serum levels; $AUC_{0\text{-}\infty}$ - area under the serum concentration-time curves from 0 h to ∞; $AUC_{3\text{-}24h}$ - area under the serum concentration-time curves from 3 h to 24 h; MRT - mean residence time.

Discussion

ABC transporters have been recognized to play an important role in drug absorption, distribution and excretion, and data on the expression of ABC transporters in different tissues may contribute to the understanding of species differences and drug-drug interactions in pharmacokinetics. As yet, only MDR1 and its gene product P-gp had been described in chickens (Edelmann *et al.*, 1999; Barnes, 2001). According to Edelmann *et al.* (1999) the homology between the deduced MDR1 protein of chickens with that of human P-gp (MDR1) reached 74%. Data regarding the expression of ABC transporters are virtually absent for turkeys, and hence the first aim of this study was to provide an overview of the expression MDR1, MRP2 and BCRP mRNA level in tissue samples of gastro-intestinal tract, liver, kidney, lung, brain and adrenal gland in healthy turkeys by RT-PCR. To this end, we sequenced relevant parts on the genes for MDR1, MRP2 and BCRP and to allow primer design and further analysis by quantitative RT-PCR. Assessment of the homology between chickens and turkey revealed a high degree of homology for all three genes (92.5 – 95.3%), and an even higher homology for the housekeeping genes beta-actin and GAPDH.

Previously, the clinical significance of drug transporters had been particularly associated with their expression at biological barriers, including the blood-brain barrier. Based on this strategic location, P-gp (MDR1) is a prominent part of the overall blood-brain barrier jeopardizing the transmission of drugs and toxins into the central nervous system. In the central nervous system, MDR1 and its protein P-gp are located predominantly on the endothelial cells of capillary blood vessels, and to a lesser degree in other cells in brain (Cordon-Cardo *et al.*, 1989; Karssen *et al.*, 2004; Mealey, 2004). Like in mammals, MDR1

mRNA was found in samples of chicken as well as turkey's brain samples. The exact cellular location could however, not be determined in the gross tissue samples. In contrast to MDR1, the levels of MRP2 and BCRP are very low. Low MRP2 and BCRP mRNAs levels were measured in rats, pigs and humans in samples of brain (Miller *et al*., 2000; Zhang *et al*., 2000; Choudhuri *et al.*, 2003; Potschka *et al*., 2003). Other biological barriers showing in mammals a high expression of efflux transporters are the placenta and the testes (Melaine *et al*., 2002; Langmann *et al*., 2003). The latter have been investigated in this study as well, and the expression of MDR1, MRP2 could be assumed, as detectable levels of mRNA for all three transporters could be found, albeit at low levels. In the interpretation of these findings it has to be considered that the used animals were at the age of 8 weeks still immature. BCRP mRNA was reported to be present in the testes of mice, rats and humans as it is in turkeys (Maliepaard *et al*., 2001; Langmann *et al*., 2003; Tanaka *et al*., 2005).

Of particular interest for the prediction of the oral availability of drugs, is the expression of efflux transporters along the gastro-intestinal tract. For **MDR1** (P-gp) the highest levels of mRNA were found in the small intestines and in the colon. Lower expression rates were observed in the caeca and the lowest in the crop and proventriculus. These data are in agreement with the observations in chickens (Edelmann *et al*. 1999; Barnes, 2001). The main difference between the results in turkeys presented here, and data obtained by Barnes (2001) in chickens, is the higher P-gp expression as compared to the colon. It is, however, noteworthy to recall that Barnes (2001) determined the tissue levels with Western blot analysis, while here we present data derived from RT-PCR studies. In mammals, including rats, pigs and humans, MDR1 expression increases from stomach to small intestines, and after decreasing in caecum, again reach high levels in the colon (Stephens *et al*., 2002; Tang *et al.*, 2004; Thörn *et al*., 2005). Next to MDR1, **MRP2 mRNA** expression is found in the gastrointestinal tract, particularly in the upper part of small intestines (duodenum and jejunum). Only low levels of MRP2 mRNA were found also in turkey's colon and caeca, similarly to the findings in chickens (Haritova *et al*., 2006; see Chapter 5). A comparative expression pattern is also found along the gastrointestinal tract of mammals (rats, mice and dogs) (Gotoh *et al*., 2000; Mottino *et al*., 2000; Conrad *et al*., 2001; Cherrington *et al*., 2002; Maher *et al*., 2005), but a much lower rate of expression is observed at least in the human duodenum, and MRP2 was not detected in the entire colon (Taipalensuu *et al*., 2001; Zimmermann *et al*., 2005). **BCRP mRNA** was found in almost similar levels trough the entire small intestines and colon, with an insignificant decrease in the caeca. These data from turkeys can not be compared with results in poultry, as BCRP has not been investigated as of yet in avian species. In rats, the expression of BCRP mRNA in the ileum and large intestines was classified as high, whereas lower levels were reported in mice (Imai *et al*., 2005; Tanaka *et al*., 2005). BCRP was expressed also at rather low transcriptional levels in the human ileum (Maliepaard *et al*., 2001). Other studies describe a maximal BCRP mRNA expression in the duodenum in humans and a decrease in

expression from the proximal to distal direction along the gastro-intestinal tract (Taipalensuu *et al.*, 2001; Allen and Schinkel, 2002; Gutmann *et al*., 2005).

In the excretory organs such as liver and kidneys, MDR1 is expressed at moderate levels in chickens (Edelmann *et al.*, 1999; Barnes, 2001; see also Chapter 5), and comparable levels were found in turkeys. In contrast, in humans and rodents, kidney levels are much higher (high levels of MDR1a mRNA and moderate of MDR1b mRNA in rodents) (Brady *et al.*, 2002). In human liver, the expression was classified as low to medium (Trezise *et al*., 1992; Langmann *et al*., 2003). Similar to chickens, in turkey's liver and kidneys MRP2 mRNA levels were higher than MDR1 mRNA. These data are comparable to the observed abundance of MRP2 mRNA in kidneys in different animal species (reviewed by Keppler *et al.*, 1998; Schaub *et al*., 1999; Faber *et al.*, 2003; Maher *et al*., 2005). In the contrary, low levels of expression were reported in dog's liver (Conrad *et al*., 2001). BCRP mRNA in turkey's liver could be classified as high, but comparable data from other avian species are not available yet. In rodent livers a high expression of BCRP is also described, whilst in humans a low rate of expression was reported (Maliepaard *et al*., 2001; Tanaka *et al*., 2005).

MDR1 mRNA was reported to be present in the lungs and trachea in mammals (Lechapt-Zalcman *et al*., 1997; Scheffer *et al*., 2002). It was detected at low levels in turkeys and chickens by RT-PCR, but was not found with Northern blotting in poultry (Edelmann *et al*., 1999). MRP2 could also be detected in the lungs of all animal species investigated, including turkeys (Torky *et al.*, 2005).

The high expression of MDR1 mRNA in adrenal glands often serves as reference in studies with human tissues (Thiebaut *et al*., 1987; Langmann *et al*., 2003). In contrast, no MDR1 was found in chickens by Northern blotting (Edelmann *et al.*, 1999), but our own results with RT-PCR indicated an expression in both, turkeys and chickens albeit at a low level (Haritova *et al*., 2006, see Chapter 5). In contrast, MRP2 mRNA could not be detected, neither in chickens nor in dogs (Conrad *et al.*, 2001), but it was found at very low levels in turkey.

In conclusion, the presented results from turkeys are in line with the tissue distribution of the three investigated ABC transporters in other species. Interspecies differences were found in the lungs and adrenal glands. MDR1 mRNA expression appeared to be comparable to MRP2 mRNA levels in the upper part of the gastrointestinal tract and the small intestines in turkeys, as described also for chickens (see Chapter 5) and mammalian species. Similar to or even higher levels of BCRP than MDR1 mRNAs were found in some parts of gastrointestinal tract in turkeys.

With the aim to assess the relevance of these findings with regard to possible differences in the kinetics of therapeutics, we conducted an experiment with different dose regimes of danofloxacin mesylate. This example was chosen in consideration of the fact that other fluoroquinolones had been identified as substrates and inhibitors for ABC transporters in other studies (de Lange *et al*., 2000; Naruhashi *et al*., 2002) and hence it had to be hypothesized that the co-localization of P-gp, MRP2 and BCRP at the apical

membranes of enterocytes limit the absorption of orally administered fluoroquinolones, as this had been described already for other drugs and toxins (Paulusma *et al*., 1996; Chan *et al*., 2004). For example, ketoconazole, a prototypic dual Pgp/CYP3A modulator and the specific inhibitor of P-gp GF 120918A increased four-fold absorption of erythromycin in monkeys by inhibition of P-gp function (Ward *et al.*, 2004; Ward and Azzarano, 2004). Moreover, inhibition of P-gp and MRPs by verapamil, quinidine, cyclosporine A and p-aminohipuric acid alter absorption of fluoroquinolones in *in vitro* and *in situ* studies (Rodrıguez-Ibanez *et al*., 2003). *In vivo* investigations with MDR1a/b (-/-) double knockout mice and MRP1(-/-) mice reveal that the disposition of grepagloxacin is at least in part governed by these two ABC transporters and that they are involved in its limited distribution to the distinct tissues (Sasabe *et al*., 2004). The functional inhibition of P-glycoprotein by cyclosporine A limited the bioavailability of grepafloxacin in rodents (Yamaguchi *et al*., 2002). Up-regulated P-gp expression and increased CYP isoenzymatic activities of small intestines in hepatic fibrosis rats may contribute to the decreased bioavailability and increased elimination of ofloxacin after oral administration (Wang *et al.*, 2006). In addition, ofloxacin competes with ciprofloxacin and pefloxacin for P-gp efflux in rat intestine which cause a significant increase in the absorption of the first fluoroquinolone drug (Rabbaa *et al*., 1997).

The second reason to select a fluoroquinolone commonly used in veterinary medicine for these model experiments in turkeys refers to the clinical practice in which systemic *Escherichia coli* infections are common and require effective therapies. The presented data indicate that Danofloxacin mesylate provokes a slight increase in the expression rates of MDR1, MRP2 and BCRP mRNA in turkey tissues, which is more prominent after continuous oral administration. Similarly, ofloxacin enhanced MRP2, as well as MDR1 mRNA expression in human colonic HCT-8 cells (Marchal *et al*., 1999). In contrast, difloxacin decreased the MRP2 expression in HL-60/AR cells (Gollapudi *et al.*, 1995). In the same experiments we also controlled the serum levels, and calculated the major kinetic parameters at the end of the first and second treatment period. These data did not show any significant differences in the results obtained with the different treatment protocols. Zhao *et al*., (2002b) also found that P-gp expression (decreased levels) and MRP2 do not influence levofloxacin pharmacokinetics in rats, treated with this fluoroquinolone and Shiga-like toxin II. Drug concentrations in the gastrointestinal tract, the liver and kidney exceeded the measured serum levels in turkeys, suggesting a role of ABC transporters in the excretion of fluoroquinolones. A comparable tissue disposition was found in pigs and rabbits (Ramon *et al*., 1994; Lindecrona *et al*., 2000). In conclusion, although repetitive administration of danofloxacin modified slightly the expression of all three measured ABC transporters, the oral bioavailability and rate of exretion of danofloxacin remained unchanged in these experiments with healthy animals. However, as disease conditions may significantly modify the levels of expression of ABC efflux transporters, further experiments should be conducted in infected animals, to verify these results.

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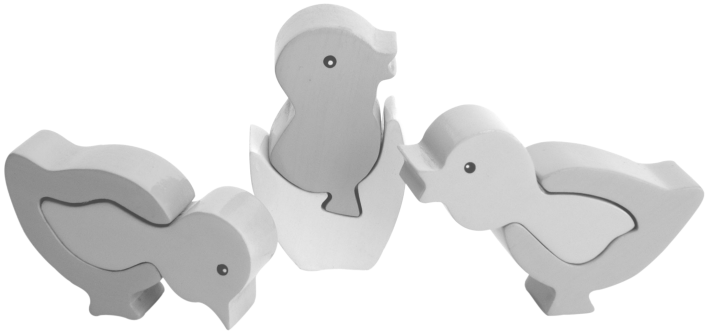
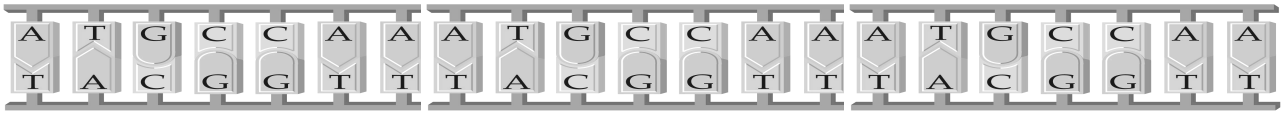
CHAPTER 8





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Effects of Fluoroquinolone Treatment on MDR1 and MRP2 mRNA Expression in Chickens Experimentally Infected with *E. coli*

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Abstract

Current knowledge about the expression of ABC transport proteins suggest that these are regulated by various factors including endogenous substances such as hormones as well a xenobiotics and pathological conditions, in particular inflammatory reactions associated with infectious diseases. As ABC transporters a major determinants of drug absorption, distribution and excretion of many antimicrobials, modulation of their activity may result in increased or decreased drug tissue levels, which consequences for the efficacy of a given treatment. As fluoroquinolones have been identified as substrates for various drug transporters, we evaluated the effect of danofloxacin mesylate and enrofloxacin treatment on the levels of expression of MDR1 and MRP2 mRNA in the intestines and liver in broilers with experimentally induced colibacillosis using an *Escherichia coli* O78/K80 isolated from a chicken farm.

The overall tissue distribution of MDR1 and MRP2 mRNA in infected chickens was similar to that in healthy birds. MDR1 mRNA expression significantly increased during the five days of oral treatment with danofloxacin mesylate and enrofloxacin in infected chickens. Changes in MRP2 mRNA expression were less prominent. The results from the presented study suggest that the treatment of colibacillosis with fluoroquinolones, which resulted in a significant clinical improvement of the animals, also restored the expression of drug transporters rather than indicating an induction by the given fluoroquinolones. Nevertheless, these obtained results are of clinical importance, as ABC transporters contribute to the integrity of the intestinal barrier as well as other important tissue barriers such as the blood-brain barrier.

Introduction

Fluoroquinolones are successfully used in treatment of colibacillosis in poultry and many investigations evaluate their efficacy according to their effects on pathogenic microorganism and the outcome of clinical studies (Ter Hune *et al*., 1991; Charleston *et al*., 1998; Chansiripornchai and Sasipreeyajan, 2002). It is also known that these drugs are not only substrates for proteins encoded by MDR1 and MRP2, but are also able to modulate the levels of expression of efflux transporters (Gollapudi *et al*., 1995; Prime-Chapman *et al*., 2005). These interactions between individual fluoroquinolones with ABC transporters were studied until now only in cell lines or in healthy laboratory animals (Griffiths *et al*., 1993, 1994; Cormet-Boyaka *et al*., 1998; Naruhashi *et al*., 2001, 2002; Rodrıguez-Ibanez *et al*., 2003), and data from avian species are completely lacking.

The current knowledge on the transcriptional regulation of ABC transport proteins suggests that infectious disease as well as other pathologic conditions impair the level and function of these transporter proteins. Previous investigations in rats and mice show a reduction of the levels of expression of MDR1 and MRP under disease conditions (Roelofsen *et al*., 1995; Vos *et al*., 1995; Trauner *et al*., 1997; Piquette-Miller *et al*., 1998; Hartmann *et al*., 2001; Sukhai and Piquette-Miller, 2000). In contrast, in chickens, the intraperitoneal injection of *E. coli* lipopolysaccharide, a common model to simulate acute infections, rapidly increased the expression of P-gp in the liver, which returned however within 24 hrs to pre-induction levels (Barnes, 2001).

Thus, infection and inflammatory diseases may provoke variability in drug bioavailability through alterations in the intestinal expression and activity of drug transporters and metabolic enzymes (Kalitsky-Szirtes *et al*., 2004).

These findings suggest an effective therapy in the course not only eliminates pathogen bacteria but also restores the expression levels and function of MDR1 and MRP2 in the liver and gastrointestinal tract, hence improving the barrier function of the intestinal tract and excretory competence of the liver, as particularly MRP2 is involved in the excretion of conjugates.

As yet, relatively little information is available regarding the *in vivo* effects of antimicrobials on MDR1 and MRP2 transcriptional levels of expression in diseased veterinary target animals, including poultry. Therefore, the effect of danofloxacin mesylate and enrofloxacin treatment on levels of expression of MDR1 and MRP2 mRNA in the intestine and liver in broilers with experimentally induced colibacillosis were investigated.

Materials and Methods

Drugs

Danofloxacin mesylate (Danocin 180, Pfizer, Part. No 4106416, 18 % sterile solution) and Enrofloxacin (Baytril 5%, Bayer AG, Part. No KP03112X, 5 % sterile solution) were used for oral treatment after being dissolved in water.

Animals

Thirty one broiler chickens (Eukarion), 3-weeks old from both sexes were obtained from a commercial poultry farm in Stara Zagora. The body weight of chickens at the start of the experiment varied between 0.6 – 0.7 kg. All animals were kept at 19-20^{o}C and had free access to standard commercial feed (without additives) and water. During the acclimatization period the animals were examined for salmonellosis and colibacillosis, but the bacterial probes were negative.

Bacterial strain and infection procedure

Escherichia coli O78/K80 strain isolated from chickens with colibacillosis was obtained from the National Scientific and Diagnostic Institute of Veterinary Medicine, Sofia, Bulgaria. The used *E. coli* strain was stored on beads at -70^{o}C prior to use. The day before the experiment *E. coli* was grown on tryptone soya blood agar (TSA; Becton Dickinson and Co, Difco Laboratories, Le Pont de Claix, France; Ref. No 236950) passaged into in Mueller-Hinton broth (MHB; Becton Dickinson and Co, Difco Laboratories, Le Pont de Claix, France; Ref. No 275730). The overnight culture was that used for the inoculation of the animals by applying 0.2 ml broth containing 2.6x10^{8} cfu/chicken intratracheally. In parallel, the inoculum was cultured on TSA for enumeration and confirmation of identity and viability of the bacteria. Clinical signs of infection appeared within 24 hours after the challenge. Three animals died during this period with pathological lesions typical for colibacillosis. The used *E. coli* O78/K80 strain was isolated from the tissues taken from experimentally infected chickens during postmortem examination to verify that this infection caused the fatalities. The MIC of danofloxacin and enrofloxacin of the strain was 0.25 μg/ml.

Study design and treatment

The infective animals were randomized in three groups. The first group (10 animals were included) was not subjected to treatment and served as a positive control group. The animals were euthanized 24 hrs after the infection and tissue samples (duodenum, jejunum, ileum, caeca, colon and liver) were taken.

The second group (10 animals) was treated with danofloxacin mesylate and the third group (8 animals) with enrofloxacin, respectively. Treatment was offered via drinking water and commenced 24 hrs after the experimental challenge and continued over a period of 5 days. The medicated water was offered *ad libitum*, and fresh batches of medicated water were prepared every 12 hrs, along with the monitoring of water intake. The concentrations of the drugs in the drinking water were checked by microbiological assay (*E. coli* ATCC 25922 as test microorganism) directly during preparation and at the end of the 12 hrs intervals. To achieve a daily dosages of approximately 6 mg/kg bw of danofloxacin mesylate, and 10 mg/kg bw enrofloxacin, the drug concentrations were adjusted according to the actual water consumption. When at the end of the experimental period water consumption of the adjusted water concentration were compared, is could be

calculated that the second group had received a dosages between of 5 mg/kg bw and 9.17 mg/kg bw danofloxacin mesylate. The third group obtained enrofloxacin in dosages varying between 10.43 and 13.95 mg/kg bw. Twenty four hours after the last dosing the animals were euthanized and tissue samples were taken from the same organs as mentioned for the positive control group. All samples stored at -70°C until being analysed.

RNA isolation

Total RNA was isolated using Trizol Reagent (Invitrogen life technologies, Cat No 15596-018). Briefly, between 50 to 90 mg of tissue was added to 1.0 ml of Trizol, placed in sterile polypropylene vials, kept on ice, and subjected to homogenisation with a power homogeniser Ultraturax (Janke & Kunkel, IKA Labortechnik). After centrifugation and incubation for 5 min at 15-30^{o}C, to the supernatant 0.2 ml chloroform were added and the samples vigorously mixed for 15 seconds followed and left at room temperature for 2 to 3 min. The vials were then centrifuged at 12,000 x *g* for 15 min. The aqueous (upper) phase containing the RNA was removed, and total RNA was precipitated during 10 min with 0.5 ml isopropanol at room temperature. After precipitation, the vials were centrifuged at 12,000 x *g* for 10 min. The supernatant was removed and the resulting pellets were washed with 1.0 ml of 75% ethanol and centrifuged again at 7,400 x *g* for 5 min. After centrifugation, the supernatant was discarded and the residual ethanol evaporated. The obtained pellet was then re-suspended in Rnase-free water; RNA concentrations were measured by ultraviolet absorbance at 260/280 nm, and stored at for a short period at -70^{o}C prior to cDNA synthesis.

cDNA synthesis

Single-stranded cDNA's are synthesized from 1 µg total RNA with the iScript^{tm}cDNA Synthesis Kit (*Bio-Rad* Laboratories, USA). To a master mixture (prepared *ex tempore*), containing 5x iScript Reaction Mix and iScript reverse transcriptase, 1 µg of total RNA dissolved in sterile nuclease free water was added. The reaction mixture was incubated for 5 min at 25^{o}C, for 45 min at 42^{o}C, followed by heat inactivation of the enzyme at 85^{o}C for 5 min and rapid cooling step towards 4^{o}C. Thereafter, the cDNA was diluted 1:10 with sterile Rnase-free water and was stored at -20^{o}C until being analysed by PCR.

Real-time PCR analysis

Primer pairs specific for MDR1 and MRP2 were designed (Table 1) and commercially produced by Isogen Bioscience BV, The Netherlands. At the same time primers for the house-keeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin genes were obtained. Ten microlitres of the diluted cDNA were mixed with 15 microlitres of iQTM SYBR Green Supermix (Bio Rad Laboratories Inc., USA), the forward and reverse primers and sterile water according to the manufacturers instructions. Real-time PCR was performed using an iCycler iQ system and MyiQ System Software

Version 1.0.410 (Bio Rad Laboratories Inc., USA). The stability of the housekeeping genes transcription under experimental conditions (treatment with danofloxacin mesylate and enrofloxacin) was tested according to the algorithm described by Radonic *et al*. (2004), using the equation $\Delta CT(t) = C_{treated} - C_{untreated}$. Gene expression was presented using an algorithms outlined by Vandesompele *et al*. (2002) and from the geNorm manual available on the web site http://medgen.ugent.be/~jvdesomp/genorm/. Dilution series of target and reference nucleic acids were used to determine the fit coefficients of the relative standard curve (efficiencies). The standards were run at the same time as the samples. Each PCR reaction included a negative control without any cDNA template. All samples were run in duplicates.

Table 1. Primers used in the PCR. The nucleotide sequences of the PCR primers used to assay gene expression by real-time PCR.

Gene	NCBI accession number	Forward primer 5' ® 3'	Reverse primer 5' ® 3'	Tm °C
MDR1	NM_204894	GCTGTTGTATTTCCTGCTATGG	ACAAACAAGTGGGCTGCTG	58
MRP2	XM_421698	CTGCAGCAAAATGAGAGGACAATG	CAGAAGCGCAGAGAAGAAGACCAC	63
GAPDH	NM_204305	GTGTGCCAACCCCCAATGTCTCT	GCAGCAGCCTTCACTACCCTCT	65
Beta-actin	NM_205518	ATGTGGATCAGCAAGCAGGAGTA	TTTATGCGCATTTATGGGTTTTGT	61

Tm – optimal annealing temperature

Statistical analysis

The results are presented as mean ± SD. All data were analysed with the Statistica 6.1 computer program (Statistica for Windows, StatSoft, Inc., USA, 1984-2002). Statistical analysis was done with the Mann-Whitney test. The non-parametric method was chosen after a test for Gaussian distribution.

Results

Clear clinical signs including depression and weakness occurred within 24 hrs after the intratracheal challenge with *E. coli* O78/K80. Gross pathological changes included a two- to three- times increase in the size of the spleen, pericarditis, imbibitions of blood vessels in the gastrointestinal tract. Mild air sacculitis was observed only in a few chickens. The most prominent changes were found in the small and large intestine: haemorrhagic enterocolitis with a large number of erosions were noticed in all infected animals, particularly in the untreated group. In the treated groups the remaining pathological alteration in the gastrointestinal tract were more severe in the enrofloxacin treated group and clinical signs persisted also longer than in the group treated with danofloxacin mesylate.

Data from the RT-PCR analysis of the house-keeping genes did not show significant differences between the three experimental groups. Moreover, GAPDH and beta-actin expression did change after treatment with danofloxacin, whereas in the enrofloxacin group an insignificant down-regulation of GAPDH was observed.

The obtained data show that MDR1 mRNA increases from duodenum to ileum, with the highest levels in the ileum. After a decrease of mRNA in the caeca, the levels are high again in the colon, and almost similar to that in the duodenum and liver. A statistically significant increase in MDR1 mRNA levels was found after treatment with danofloxacin ($P<0.0003$-0.01) and enrofloxacin ($P<0.002$-0.03) in almost all tissues. (Fig. 1).

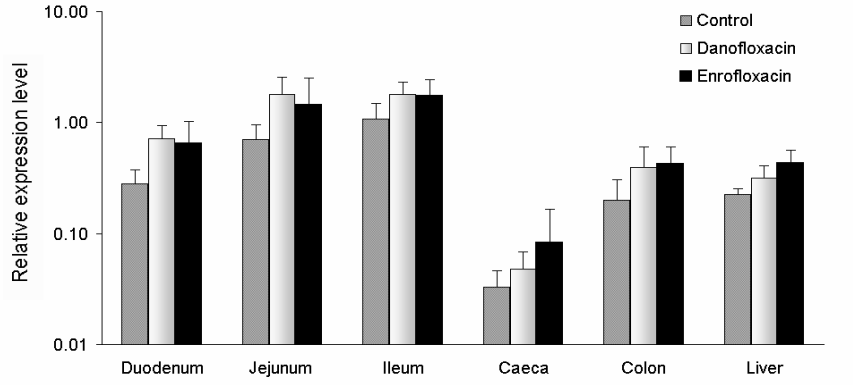

Figure 1. Relative levels of expression of MDR1 mRNA normalized against GAPDH and beta-actin in chickens, experimentally infected with pathogen strain *E.coli* O78/K80. Tissue samples from the positive control group (n=10) were obtained 24 hrs after the challenge. Tissue samples from the chickens treated orally with danofloxacin mesylate (n=10) and enrofloxacin (n=8) for 5 consecutive days starting 24 hrs after the challenge, were taken on day 6.

MRP2 mRNA was found predominantly in the small intestines, but not in the caeca. Levels in the colon were very low and at the limit of detection. MRP2 mRNA levels in the liver were as high as in the duodenum. Treatment with danofloxacin mesylate resulted in an increase in MRP2 mRNA in the duodenum and jejunum. In contrast, levels in the ileum and colon remained low, and a significant down-regulation was observed in the liver ($P=0.04$) and colon ($P=0.02$). No changes or almost a slight tendency towards up-regulation was found in the investigated tissues after treatment with enrofloxacin, with a statistically significant increase of levels of expression of MRP2 mRNA ($P=0.016$) only in the jejunum (Fig. 2).

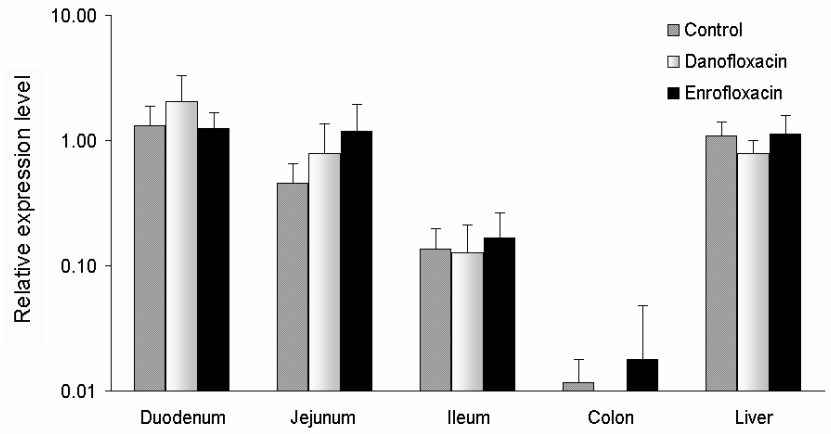

Figure 2. Relative levels of expression of MRP2 mRNA normalized against GAPDH and beta-actin in chickens, experimentally infected with pathogen strain *E.coli* O78/K80. Tissue samples from the positive control group (n=10) were obtained 24 hrs after the challenge. Tissue samples from the chickens treated orally with danofloxacin mesylate (n=10) and enrofloxacin (n=8) for 5 consecutive days starting 24 hrs after the challenge, were taken on day 6.

Discussion

Exposure to pathogens activates a series of defence mechanisms, generally described as acute phase response to infection. Typical for this response is the expression of cytokines, in particular tumour necrosis factor alpha (TNFα), interleukin (IL) 1β and IL-6, which are the most prominent pro-inflammatory cytokines, found also in chickens during an acute phase response, albeit that differences in the cytokine mRNA expression was found in broilers as compared to layers, which may explain the divergence in LPS responsiveness (Leshchinsky and Klasing, 2001). In mammals, the expression of pro-inflammatory cytokines is accompanied with a down-regulation of the nuclear receptors such as FXR (Farnesoid X receptor), PXR (Pregnane X Receptor), and CAR (Constitutive Androstane Receptor) which are involved in the transcriptional regulation of ABC transporters (Beigneux *et al*., 2002; Kim *et al*., 2003; Stienstra *et al*., 2004; Geier *et al*., 2005). Subsequently, a number investigations aimed at defining the role of individual cytokines in the transcriptional and posttranscriptional regulation of the activity of MDR1 and MRP2 (McRae *et al*., 2003; Siewert *et al*., 2004). For example, it was found that P-gp (MDR1) is down-regulated by pro-inflammatory cytokines in a concentration-dependent manner. Moreover it could be shown that IL-1β acts apparently at the level of the protein, whereas IL-6 affects P-pg expression at the transcriptional level (Sukhai *et al*., 2000, 2001). Endotoxins of Gram-negative micro-organisms down-regulate simultaneously hepatic P-glycoprotein, MRP2 and CYP3A isozymes (Nadai *et al*., 2001; Zhao *et al*., 2002; Ueyama *et al*., 2005). In rodents a decrease of mRNA and protein levels of P-gp was observed also

in the intestines, particularly in the large intestines, as well as in the liver and brain. These changes resulted in altered pharmacokinetics of P-gp substrates (Piquette-Miller *et al*., 1998; Veau *et al*., 2002; Goralski *et al*., 2003; Iizasa *et al*., 2003; Sun *et al*., 2006).

In contrast to the considerable number of investigations in mammalian species, particularly in rodent, studies with avian species are virtually lacking. The only example is a study of Barnes (2001) demonstrating that in chickens, the expression of P-gp protein in the liver rapidly increased following a single LPS injection, but declined to pre-induction levels within 24 hours. No changes were observed in the duodenum (Barnes, 2001). In contrast, in mice the expression of P-gp was significantly decreased 6 h after an endotoxin injection, but returned to the control levels also within 24 h (Miyoshi *et al*., 2005). Moreover, the expression of MRP2 mRNA was down-regulated in the liver and intestines during an acute inflammatory response in mice and rats (Tang *et al*., 2000; Geier *et al*., 2002; Hartmann *et al*., 2002; Kalitsky-Szirtes *et al*., 2004; Teng and Piquette-Miller, 2005). In inflammation-induced cholestasis, no changes in MRP2 mRNA levels were observed in the human liver, although the MRP2 protein level was markedly reduced, which suggest posttranscriptional regulation (Zollner *et al*., 2001; Elferink *et al*., 2004; Geier *et al*., 2005). Taken together, these findings indicate not only the existence of a species-specific response to increased levels of pro- and anti-inflammatory cytokines in the course of an inflammatory reactions, but suggest also that individual cytokines interfere with the regulation of ABC transporters at the transcriptional and post-transcriptional level.

An altered expression of efflux transporters may account for some of the observed differences in kinetics between healthy and diseased animals. For example, a decreased P-gp expression in the intestines may increase the membrane permeability for drugs, but also for toxins. Down-regulation of P-gp and CYP450 3A, which is one of the major cytochrome expressed in enterocytes, seriously impairs the barrier function of the intestinal wall, while at the same time increasing the oral bioavailabily of various drugs (Haghgoo *et al*., 1995; Nadai *et al*., 1998; Bertilsson *et al*., 2001). Administration of dexamethasone prior to an LPS challenge, decreased the release of cytokines, and reversed the reduction of MDR1a, MDR1b, OATP1, OATP2, OCT1, and NTCP mRNA (Cherrington *et al*., 2004). Pre-treatment with polymyxin B reduced plasma endotoxin levels in the portal blood, prevented cholestasis, and changes in immunolocalization of MRP2 in rats. In contrast, neither penicillin G nor metronidazole or clindamycin (which are obviously not substrates for ABC transporters and do not change LPS levels) prevented the cholestasis in rats with colitis (Yamada *et al*., 1996; Kawaguchi *et al*., 2000). The significance of MDR1a in the protection against colitis was also demonstrated in MDR1 knock-out mice (Panwala *et al*., 1998; Fromm *et al*., 2002). Spontaneous development of colitis in these mice was significantly higher than in wild type mice.

At the same time there are examples which show that the increase of P-gp activity after treatment of *Helicobacter pylori* infection with an antibiotic could be a reason for a failure of therapy during a second therapy with the same or another antibiotic (Babic *et al*., 2005). These findings prompted us to measure the levels of expression of MDR1 and

MRP2 in the intestines and the liver of chickens suffering from *E.coli*-induces hemorrhagic enterocolitis. The obtained data suggest an increase in the levels of transcription during the therapy. These findings may, however, be misleading, as no true negative controls were included in the experimental design. Hence it can not be excluded that the given *E.coli* challenge primarily resulted in a down-regulation of both efflux transporters, and that the measured increase under therapy reflects the restoration of the levels of expression along the reduction of infectivity due to the antimicrobial treatment, rather than indicating a direct effect of the given fluoroquinolones. Moreover, it has to be taken into account that the positive control group was analysed already 24 hrs after the *E.coli* challenge, at a time were serious clinical signs indicated the acute phase response to the infection. In contrast, samples from treated animals were obtained 6 days after the challenge and at this time the animals had surpasses the acute phase response, and had clinically improved. Although on the basis of the presented data in remains unclear to what extend the observed increase in the levels of MDR1 transcription has to attributed to a direct effect of the given fluoroquinolones, or to the fact that these effectively combated the infection an allowed for restoration of transcription, the measured effect is benifical for the animal, as this up-regulation improves the barrier function of the gastro-intestinal tract (Panwala *et al*., 1998; Sakaeda *et al*., 2002; Schwab *et al*., 2003; Leslie *et al*., 2005). In the liver, particularly the increase in the level of MRP2 is considered as beneficial, as this transporter is essential for the efflux of conjugated, including glucuronides glutathione conjugates, sulfate conjugates. More importantly also bilirubin can only be excreted in the form of its glucuronide, and hence a normal or induced level of MRP2 protect hepatocytes from oxidative damage induced by increasing amounts of free bilirubin, and subsequent cholestasis. The apparently lower expression of MRP2 mRNA in the liver following the application of danofloxacin mesylate requires further investigations, as an impairment of MRP2 functionality would decrease of the ability to eliminate its physiological conjugates as well as drugs and their conjugated metabolites. Further experiments at the functional level are needed to determine the clinical significance of these changes.

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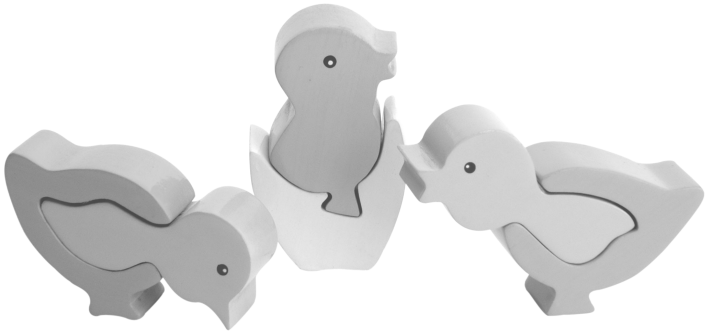
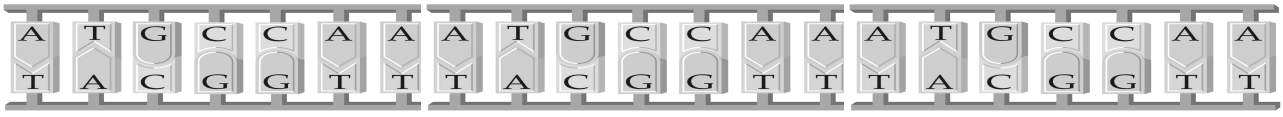
CHAPTER 9





9

General Discussion

This thesis addressed two main areas of interest in poultry medicine. In the first part, advance kinetic models (PK-PD), based on an integration of pharmacokinetic and pharmacodynamic (antimicrobial activity) data are presented, that are considered presently as the most optimal approach to validate dose regimens of fluoroquinolones. Although comparable data have been published for other animal species, kinetic data in poultry species were limited, and virtually lacking in turkeys. As an indication of a further refinement of kinetic analysis, we tried to implant tissue chambers subcutaneously. Again, this method had been applied before in a different form in larger animals, including horse, calves and sheep, with the aim to have an ethical model to study inflammatory responses to infections, as well as the kinetics and efficacy of antibiotics that need to reach the interstitial space. This model meets all ethical requirements as any infection and/or inflammatory response is confined to the tissue cage, and hardly affects the health and welfare of the animal. The presented pilot study clearly indicates, that this model can be easily adopted for studies in poultry, provided that smaller (tailor made) tissue chambers are used.

The second part of the thesis addresses membrane transporters, belonging to the ABC binding cassette of transporters that use the energy of ATP hydrolysis to transport a wide range of substrates across biological membranes. Discovered as multi-drug resistance proteins and efflux transporters that explained therapy-resistance of cancer cells following long-term applications of cytostatic agents, these membrane transporters are now recognized to affect the absorption, distribution and elimination of a wide range of drugs. Moreover, increasing evidence suggest their role in the pathogenesis of important diseases, including various inherited familiar liver diseases. In contrast to the field of human medicine, in which these ABC transporters are intensively studied, the number of investigations in veterinary medicine is still very limited, and data in avian species are virtually lacking as yet. Hence we developed a series of RT-PCR methods to determine the expression in various organs of chickens and turkeys, and to measure the effects of fluoroquinolones on the level of expression in healthy and diseased animals.

Pharmacokinetic/Pharmacodynamic approach of fluoroquinolones in poultry

Fluoroquinolones are widely used in poultry husbandry to control infectious diseases (Glisson *et al.*, 2004; Huff *et al*., 2004). However, little is known about pharmacokinetics of danofloxacin mesylate and marbofloxacin in turkeys and published data describe their disposition mainly in chickens (Knoll *et al*., 1999; el-Gendi *et al*., 2001; Anadón *et al*., 2002). Their clinical efficacy has been tested in chickens and turkeys (Ter Hune *et al*., 1991; Charleston *et al*., 1998) but increasing concerns were expressed about the abundant use of these drugs in poultry, as resistant zoonotic bacteria may reach the consumer (Humphrey *et al*., 2005). It has been shown that in mammals the PK-PD approach (the combination of pharmacokinetic data with phramacodynamic parameters) is a valuable tool in selection of optimal drug dosages with the aim to prevent the appearance of antimicrobial resistance (Thomas *et al*., 1998). Therefore, these PK-PD approaches have been tested in poultry, with the aim to propose optimal dosing regimes for further clinical trials (**Chapter 2 and Chapter 3**). Our findings show that pharmacokinetic properties of danofloxacin mesylate and marbofloxacin after i.v. administration in turkeys are similar to that in chickens (Anadón *et al*., 1997; Knoll *et al*., 1999; Anadón *et al*., 2002). Following oral application, both fluoroquinolones are slowly absorbed to a high extend in turkeys, with a higher bioavailability than in chickens. In turkeys, the double peak of the serum concentrations, found after oral administration of danofloxacin might be related to the inhibitory activity of danofloxacin on P-gp function (**chapter 5**). These double peaks in serum concentrations were also observed in broilers after oral administration of enrofloxacin (Sumano *et al*., 2003). Hence, further investigations should address the pharmacokinetics of danofloxacin administered simultaneously with a specific inhibitor for P-gp in turkeys, and studied in an *in situ* model based on isolated intestinal loops from poultry.

The most frequently used pharmacodynamic index for measuring the activity of an antimicrobial *in vitro* is the estimation of the MIC, and according to this parameter marbofloxacin is less active than danofloxacin mesylate against the investigated pathogenic *E. coli* O78/K80 strain. Both drugs showed reduced activity when tested in the presence of serum, and these data confirmed previous findings (2 to 4 fold higher MIC values; Jacobs *et al*., 2002). The reasons for these differences remain to be elucidated but may reflect differences in the growth rate of bacteria under different culture conditions (Mouton and Vinks, 2005). The antibacterial activity of marbofloxacin against *E. coli* O78/K80 in serum appeared to be slower during the first six hours of incubation, as compared to danofloxacin.

AUC_{24}/MIC_{50} is a surrogate marker, which has a high predictable value for the outcome of therapy following the administration of fluoroquinolones. Our findings suggest that bacterial elimination could be achieved with danofloxacin at lower *ex vivo* AUC_{24}/MIC ratios as compared to marbofloxacin. Concentration-dependent killing action of *E. coli* O78/K80 is suggested at relatively high Hill coefficients. The envisaged value of C_{max}/MIC

suggesting 99% reduction in bacterial counts could be only achieved with danofloxacin. The results for marbofloxacin and previously published data for enrofloxacin in turkeys suggest a lower killing rate (higher survival) of pathogens and hence the risk for the development of antimicrobial resistance against fluoroquinolones remains (EMEA, 1999; Giraud *et al.*, 2001; Haritova *et al.*, 2004). Marbofloxacin, however, possess some preferable pharmacokinetic properties in comparison to other fluoroquinolones such as low serum protein binding and Cl_B, which should compensate for the lower activity against *E. coli* O78/K80 (**Chapter 3**). The findings in **chapters 2 and 3** show, that in turkeys both fluoroquinolones could be appropriate choice to achieve clinical cure of *E. coli* infections. Following the paradigm that the AUC/MIC ratio should exceed a value of 125, reaching under optimal conditions even a ratio of 400-500 (Forrest *et al*., 1993; Hyatt *et al*., 1994), the data presented would suggest optimal oral doses of 3.0-12.0 mg/kg bw per day for marbofloxacin, and 7.7-32 mg/kg bw per day for danofloxacin mesylate. These suggestions are in line with the proposed higher dose regimens for enrofloxacin, sarafloxacin and norfloxacin in turkeys (Laczay *et al.*, 1998).

Tissue chambers were introduced as a model to study interstitial fluid concentrations of antimicrobials in PK-PD investigations of antibiotics, the inflammatory responses and the anti-inflammatory effects of different drugs in various animal species, but not in birds (Greko *et al*., 2003; Aliabadi and Lees, 2003; Werners, 2005). **Chapter 4** described a possibility to implant the tissue cages in turkeys with the aim to obtain samples of interstitial fluid. This pilot experiment offers broad possibilities to study the kinetics and typical pharmacodynamic characteristics of antimicrobials and other pharmaceuticals in the living animal.

ABC transporters in poultry

ABC genes encode a super family of ATP-binding cassette transporters which are transmembrane proteins that can carry a wide variety of substrates across biological membranes in an energy dependent manner. These transporters have been extensively studied in humans and laboratory animals since P-glycoprotein was described first by Juliano and Ling (1976). MDR1 (P-glycoprotein), MRP2 and BCRP are efflux proteins, which can play an important role in pharmacokinetics of a large range of drugs, in drug-drug interactions and in preventing the uptake of toxic compounds (Yu, 1999; Trauner and Boyer, 2003; reviewed in **chapter 1**). They respond to influences of these drugs, as well as to diseases and inflammatory conditions, dietary components and a wide range of endogenous substances (Tanaka *et al.*, 2002). Increasing evidence demonstrates that fluoroquinolones are among drugs that are substrates and inhibitors of ABC transporter proteins in mammals (de Lange *et al.*, 2000). It was also demonstrated that the levels of expression and the functional activity of these proteins differ between animal species (Annilo *et al.*, 2003), limiting the possibility to extrapolate data between animal species. Only a very limited number of investigations address the expression of P-gp (and other

ABC transporters) in avian species (Edelmann *et al*., 1999; Barnes, 2001) and no information has been published from turkeys. The present work describes the levels of mRNA of MDR1, MRP2 and BCRP in poultry and evaluates influence of fluoroquinolones on their expression in healthy and infected animals. Moreover, some functional studies are presented that may serve as screening assays in the identification of substances that are substrates for these transporters and also affect their functional activity.

Fluoroquinolone drugs are synthetic antibacterial agents with activity against Gram-negative and Gram-positive pathogens (Van Bambeke *et al*., 2005) and because of their advantageous pharmacokinetic and pharmacodynamic properties and low toxicity they are widely used in the oral treatment of infectious diseases in poultry (Jordan *et al.*, 1993; Laczay *et al*., 1998). Although, their pharmacokinetic behaviour has been described in many species of veterinary interest, their kinetics, especially in relation to their pharmacodynamics, is less investigated in poultry and data from turkeys are lacking as yet. This implies that the concept of PK-PD modelling, often used to optimize dose regimens for fluoroquinolones, has been not applied in poultry species (Lees and Aliabadi, 2002). Therefore, the possibilities to use this approach in poultry were tested in the first part of the thesis.

Comparative aspects in chickens and turkeys.

ABC genes are found in almost all living organisms, ranging from microorganisms and fungi to higher classes of vertebrates (Van Veen and Konings, 1998; Dean and Annilo, 2005). MDR1, MRP2 and BCRP are members of this family, which are extensively studied because of their role in the pharmacokinetics of wide range of drugs and their involvement in the transport of physiological substrates in mammals (Johnstone *et al*., 2000; Adachi et al., 2005). Although, these genes are conserved in all vertebrates, there are also several examples of duplications and deletions, resulting in genetic instability within species (Kim *et al*., 2001) and differences between species (Annilo and Dean, 2004). In line with this previous observations, sequence analysis of conserved regions of the genes encoding for transporter proteins, show that the homology between chickens and turkeys (approximately 95%) is much higher than between chicken and human MDR1 (74%). In poultry also MRP2 and BCRP were found in various tissues (**Chapters 5 and 7**).

MDR mRNAs and P-gp levels of expression in ***gastrointestinal tract*** were extensively documented in laboratory animals and humans (Brady *et al*., 2002; Langmann *et al*., 2003). Most of the studies reported that the levels of MDR1 mRNA increased from stomach to ileum and again in the colon. Northern and Western blot (Edelmann et al., 1999; Barnes, 2001) and RT-PCR analysis show that MDR1 and MRP2 expression in chickens (**Chapter 5**) and in turkeys (**Chapter 7**) revealed no substantial differences in their levels of expression in the gastrointestinal tract. However, whereas in mammals, the highest levels of BCRP were found in ileum and in large intestines, in turkeys BCRP expression was

found to be similar in the entire intestinal tract and was even higher in some parts than the expression of MDR1 mRNA. A significant expression of MDR1 mRNA in ***liver***, albeit still lower than in intestines, is typical for mammals and poultry (Edelmann *et al*., 1999; Merino *et al.*, 2005). In contrast, MRP2 and BCRP mRNAs levels were higher in the liver in comparison with MDR1, reflecting their role in the bilary excretion of endobiotics and xenobiotics (Dietrich *et al*., 2001).

MDR1 and BCRP mRNAs are present at moderate to high levels also in the ***kidneys*** in mammals as well as in chickens and in turkeys as demonstrated in our studies, hence confirming previous results (Edelmann *et al.*, 1999; Langmann *et al*., 2003; Tanaka *et al*., 2002).

Data on the expression of MDR1 and BCRP in the ***lung*** remains inconsistent. The presented results from turkeys and chickens show that MDR1 mRNA is present only at low levels in the lungs, whereas Edelmann *et al.* (1999) found higher levels by Northern blotting, which might be attributable to the methodical differences, but also to strain differences in the breed of animals used. BCRP was found at higher levels in turkeys, whereas it was defined as detectable (thus at low concentrations) in human lung tissue (Scheffer *et al*., 2002).

The functional role of P-gp, the product of MDR1 genes, has been initially related to the function of the blood-brain barrier, in limiting the penetration of potentially toxic compounds into the ***brain*** in humans, rodents and dogs (Cordon-Cardo *et al*., 1989; Mealey *et al*., 2003; Karssen *et al*., 2004). Detection of MDR1 and BCRP mRNAs levels in poultry brain (Edelmann *et al.*, 1999; **chapters 5 and 7**) is most likely related to their expression in the capillary endothelium (Maliepaard *et al*., 2001). MRP2 has been found also in the brain of fish, rats, pigs and humans (Choudhuri *et al.*, 2003; Potschka *et al*., 2003), but not in canine and bovine brain capillary endothelial cells (Zhang *et al*., 2000; Conrad *et al*., 2001). MRP2 mRNA was not detected in brain of chickens but detectable levels in turkeys were found.

It has been shown that MDR1 in mammals is essential for the function of ***adrenal glands*** (Langmann *et al.*, 2003) but according to our results (**chapters 5 and 7**) and these by Edelmann *et al.* (1999), MDR1 and MRP2 were not detected in the organs of chickens and were found at low levels in turkeys. However, BCRP mRNA is present at high levels in turkeys. The reason for such a difference still remains to be clarified.

MDR1 and BCRP were defined as efflux proteins involved in tissue barriers including those protecting ***ovaries*** and ***testes*** in mammals and mature chickens (Edelmann *et al.*, 1999; Melaine *et al.*, 2002; Langmann *et al.*, 2003). Low levels of MDR1 mRNA, but higher levels of BCRP mRNA (**chapter 7**) were found in immature birds as compared to previous published results from adult animals.

In conclusion, the presented findings support the hypothesis defined by Dean and Annilo (2005) that ABC transporters are highly conserved during the evolution and present in all animal species, albeit with subtle, but often clinically relevant differences is their

expression in individual organs. The results obtained in our study from chickens and from turkeys could be compared only with data in rodents and humans, as literature about ABC transporters in other species is very limited and confined to very few studies in dogs, pigs and preliminary studies in poultry (Conrad *et al*., 2001; Tang et al., 2004; Geyer *et al*., 2005). The individual level of expression in poultry may change in accordance with age, drug administration and disease conditions (Kim *et al*., 2000; Barnes, 2001; Chen *et al*., 2005).

ABC transporters and fluoroquinolones

One of the classes of antibiotics that has been identified to be substrates of ABC transporters are the fluoroquinolones. It is worthwhile to mention that fluoroquinolones not only are transported across biological barriers, but also may modulate the levels of expression of individual efflux proteins. This observation is not only made in target animal species and human, but strikingly, is one of the basic mechanisms of antibacterial resistance associated with the use of fluoroquinolones in bacteria, in which the up-regulation of P-gp-like transporters conveys resistance to these antimicrobial drugs. In consideration of emerging problems with antimicrobial resistance, modern fluoroquinolones are now recommended at dose regimens, meeting the principles of concentrations-depending dosing, with the aim to achieve an absolute bactericidal concentration within the target organs, to reduce the risk of resistance development. In turn, various attempts were made to establish an optimal dose-regimen for these drugs. A widely recognized concept is PK-PD modeling, which allows to assess and compare individual fluoroquinolones and their pharmaceutical formulations. As mentioned above, these investigations have been confined in the past to major farm animal species, but did not include any poultry species. Therefore we presented in **Chapter 2** and **Chapter 3** original investigations applying the PK-PD concept to experimental studies conducted with danofloxacin mesylate and marbofloxacin in turkeys.

Effect of fluoroquinolones on the levels of expression of ABC transport proteins in healthy and diseased animals

Several investigations showed that an up-regulation of P-gp, MRP2 and BCRP can impair the outcome of a therapy, because of the subsequent changes in drug absorption and disposition (Gustafson and Long, 2001; Honjo *et al*., 2001; Dantzig *et al*., 2003). The most prominent examples, are cytostatic agents used in cancer therapy. They were among the first substances found to be able to modulate the expression of ABC transporters at cellular membranes. Together with the fact that many cancer cell types increase during de-differentiation also the level of efflux transports, this resulted in the well-known reduction in efficacy of cytostatic agents during long term therapy.

Some antimicrobials, including fluoroquinolones, are obviously able to change the levels of expression of MDR and MRP mRNAs as well (Gollapudi *et al*., 1995; Salphati

and Benet, 1998; Schrenk *et al*. 2001). For example, increasing levels of expression of MDR1 and 2, and MRP2 mRNA were observed after administration of ofloxacin, erythromycin and rifampicin in different mammalian species (Gant *et al*., 1995; Fromm *et al*., 2000; Schrenk *et al*. 2001). In contrast, MRP mRNA levels were decreased in HL-60/AR cells exposed to difloxacin (Gollapudi *et al*., 1995).

Notwithstanding, that there is some information about the modulation of MDR1 and MRP2 in mammals following fluoroquinolone treatment, no comprehensive data are available in poultry. Therefore, we studied the influence of an oral treatment of healthy and infected animals with danofloxacin mesylate on mRNA levels of MDR1, MRP2 and BCRP.

Danofloxacin mesylate, like ofloxacin, provoked a minor, statistically insignificant increase in all investigated genes in tissues of healthy turkeys. This increase became more prominent after continuous oral administration, but did not cause alterations in serum levels and pharmacokinetic parameters of orally administered danofloxacin mesylate (**chapter 7**). These findings suggested that the risk for drug-drug interactions is rather low with such a therapy, but further investigations at functional levels should confirm these results.

No only chemical substances but also inflammatory reactions and the release of cytokines modify the expression of ABC transporters (Laouari *et al*., 2001; Fernandez *et al*., 2004; Geier *et al*., 2005). It is worthwhile to reiterate in this context, that one of the first specific inhibitors of P-gp that emerged in mechanism-based research was LTC4 (a leukotriene analogue). Endotoxin of Gram-negative microorganisms was shown to down-regulate simultaneously hepatic P-glycoprotein and MRP2 (as well as CYP3A) and to impair the transport of their substrates (Ando *et al*., 2001; Ueyama *et al*., 2005). This down-regulation increases the risk for absorption of (bacterial) toxins from the intestines and impairs liver function to a decrease in the excretion of bile acids, hence accelerating the disease status (Schwab *et al*., 2003; Leslie *et al*., 2005). With our own results, we could demonstrate that treatment with two different fluoroquinolones that are commonly applied in veterinary practice results in a significant restoration of physiological MDR1 expression, although the levels of healthy chickens were not completely regained (**Chapters 8 and 5**). This restoration of MDR1 expression under therapy seems to reflect the efficacy of the given fluoroquinolones to combat the infection, resulting in a decreased LPS burden, hence decreasing the levels of pro-inflammatory cytokines. The expression of MRP2, was not significantly effected by the infection. However, also for MRP2, a tendency towards an increase in the expression following the treatment with fluoroquinolones could be observed. In contrast, a significant decrease of MRP2 mRNA in the liver after treatment with danofloxacin mesylate was measured. Taken together, these data suggest that under an effective therapy, the expression of MDR1 and hence the level of P-gp is gradually restored, which is of benefit to the animal, as P-gp contributes to an effective intestinal barrier.

Fluoroquinolones as inhibitors of P-gp: functional studies

Flow cytometry has been introduced as an elegant method for the analysis of the functional activity of ABC transport proteins in cell lines (*in vitro*) and in PBMCs (*ex vivo*) (Canitrot *et al*., 1996; Steinbach *et al*., 2002). This method has been widely applied in mammalian cells to investigate the function of efflux proteins (Klimecki *et al*., 1994), but is described here for the first time for cells obtained from poultry (**Chapter 6**). When evaluating the existing protocols for their appropriateness for poultry studies, it was evident that this was not readily possible as any fraction of PBMCs, even after Ficoll separation, contained a very high number of thrombocytes (Ries *et al*., 1984). Moreover, the isolation of peripheral mononuclear cells (which is theoretically possible by quantitative FACS analysis) results in a very limited number of cells that can be obtained from smaller animals (young chickens), which might to be sufficient to conduct comparative studies with different compounds or for testing a range of concentrations of an individual drug. Therefore, we preferred to use cell suspension of splenocytes, which contains almost exclusively $CD3^+$ and $CD8\alpha^+$>$CD8\beta^+$ and $CD4^+$ positive cells and less than 5% thrombocytes (see also Lacoste-Eleaume *et al*., 1994). In this primary cell suspension, Rh-123 uptake and excretion was measured by flow cytometry (**Chapter 6**). Initial experiment indicated that in these cells Rh-123 efflux is readily saturated at higher substrate concentrations, a finding observed in other model experiments as well (Bains and Kennedy, 2005). Subsequently, in all following experiments, low concentrations of Rh-123 (0.125 and 0.5 μM) were used.

To elucidate the contribution of individual transport proteins in Rh-123 efflux, various known inhibitors were tested. Previous experiments in mammalian cells had indicated that cyclosporin is a broad inhibitor of various membrane transporters; PSC 833 was found to be a rather specific inhibitor for P-gp (Qadir *et al*., 2005), and GF 120918 was found to be a highly specific inhibitor for P-gp at low concentrations as well, whereas at higher concentration it inhibits BCRP as well in mammalian cells (Hyafil *et al*., 1993). The fact that in our experiments with avian splenocytes only PSC 833 and GF 120918 could modulate Rh-123 excretion points to the pivotal role of P-gp expression in this cell type. GF 120918 was found to be the most potent inhibitor but its efficacy did not approach 100%. This finding suggests that passive diffusion of Rh-123 also plays a role in its extrusion from the cells. Like in other species, two separate clusters with different P-gp activities were identified in isolated avian splenocytes (Chaudhary *et al*., 1992; Klimecki *et al*., 1994), which should be characterized in more detail in forthcoming experiments. In contrast to P-gp, very low levels of MRP2 and BCRP expression were found in chicken splenocytes, as demonstrated by inhibition studies with MK 571 and Ko-134 (**Chapter 6**).

In these studies with avian splenocytes, marbofloxacin sulfate, enrofloxacin and ciprofloxacin did not affect Rho-123 transport significantly. A slight inhibitory effect was observed when danofloxacin and danofloxacin mesylate were tested, but this effect corresponded only to approximately 10% of the inhibitory effect of GF 120918. It was

concluded that the tested fluoroquinolones, which are commonly used in veterinary practice, seem to be only weak inhibitors of P-gp mediated transport processes. It needs, however, to be elucidated if these fluoroquinolones exert an inhibitory effect on other efflux transporters, as in the isolated splenocytes the expression of MRP2 and BCRP was too low to quantify any inhibitory effect.

Summarising conclusion and future perspectives

In conclusion, the findings of the presented studies related to the pharmacokinetics of fluoroquinolones in chickens and turkeys indicated that PK-PD models could be applied in poultry as well to suggest dosage regimens for forthcoming clinical trials. It should, however, be mentioned that any PK-PD approach has its clear limitations. A remaining variable is the varying intrinsic sensitivity of field isolates of *E. coli* against danofloxacin and marbofloxin. In our approach the PD data (i.e. MIC and MBC values) were determined only with one individual strain, which might not have been representative. Other factors known to influence the outcome of treatment are: the immune status of the animals before and during the infection, (patho-)physiological changes during infection, and the penetration of the drug into the infected tissue or organ. These factors are inadequately addressed in a PK-PD model that is based only on drug concentrations in serum or plasma. The possibilities to use tissue chambers and to measure interstitial drug concentrations as well as the decline of bacterial counts following treatment of test infections (as well as mediators of inflammatory reactions), indicate the advantages of this model. We could demonstrate with a pilot experiment that tissue chambers can be also used in poultry. This opens the possibility to incorporate this type of experiments in forthcoming PK-PD trials.

In the second part of the thesis we demonstrated that efflux proteins, encoded by MDR1, MRP2 and BCRP genes, are expressed in poultry species (chickens and turkeys) as well. Their level of expression and functional activity could be modulated by fluoroquinolones, used as model compounds, and by iatrogenic infections with *E.coli*. The tissue localization of MDR1, MRP2 and BCRP resembles that described in mammals, suggesting similar functions in drug absorption, distribution, metabolism and excretion, albeit with quantitative differences between individual animal species. Further investigations of ABC transporters should identify possible interspecies, breed, sex and age related differences in their levels of expression in poultry, as these could be of clinical significance.

The significance of the participation of these efflux proteins in drug-drug interactions and in pharmacokinetics of substances, widely used in poultry husbandry, is sill not fully understood. The same applies to the effect of these drug transporters as modulators of the internal dose and the route of excretion (including eggs) of toxins and other contaminants of animal feeds. The presented investigations in infected chickens suggest that more attention should be paid to the effects of bacterial endotoxins and viral infections,

as well as inflammation (and the release of inflammatory mediators such as cytokines) on the levels of expression and function of ABC transporters. Since these proteins serve as a barrier at different sites of organism, they have not only a protective role, but might serve also as targets to improve the penetration of drugs or vaccines into target tissues.

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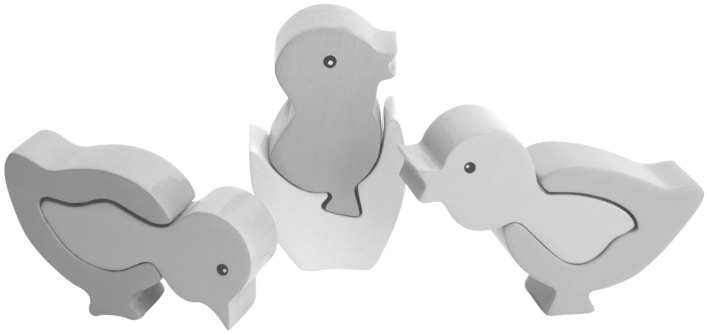
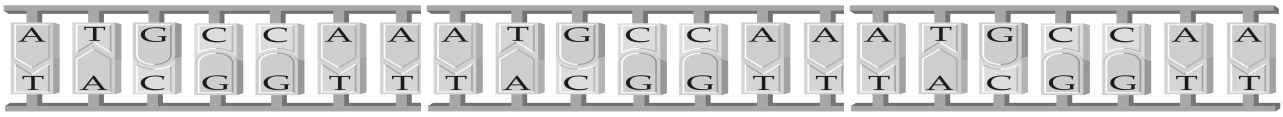
NEDERLANDSE SAMENVATTING





Het onderzoek, dat in dit proefschrift beschreven wordt, richt zich op twee aspecten die van belang zijn in de pluimvee geneeskunde. Allereerst wordt ingegaan op de koppeling van farmacokinetische- en farmacodynamische gegevens (PK-PD modeling) ter optimalisering van doseringsvoorschriften voor fluoroquinolonen. Om de farmacokinetische studie te verfijnen kan gebruik gemaakt van onderhuids geïmplanteerde weefselkamertjes. Het tweede deel van het proefschrift gaat nader in op de rol van membraantransporteiwitten betrokken bij de opname, verdeling en uitscheiding van fluoroquinolonen. Deze, tot de "ABC binding cassette" behorende transporters, zijn voor hun energiebehoefte afhankelijk van ATP-hydrolyse, en transporteren geneesmiddelen en toxische stoffen, maar ook lichaameigen stoffen, zoals bijvoorbeeld galzuren. Zij spelen een belangrijke rol in de bescherming van gevoelige weefsels, en zijn mede voor de functie van de blood-hersen-barrière, of de placentabarrière verantwoordelijk. Tevens spelen zijn blijkbaar ook een rol bij ontstekingsprocessen en de pathogenese van belangrijke aandoeningen. Om dit nader te onderzoeken werd een aantal RT-PCR methoden ontwikkeld om de expressie van deze transporters in verschillende organen van kippen en kalkoenen na te gaan. Tevens werd bij gezonde en zieke dieren de mate van expressie onder invloed van fluoroquinolonen gemeten.

Fluoroquinolonen in de pluimveegeneeskunde: een farmacokinetische-farmacodynamische benadering

In de pluimveesector worden fluoroquinolonen frequent ingezet om bacteriële infecties te bestrijden. De farmacokinetiek van deze antibiotica is vooral bij kalkoenen onvoldoende bestudeerd. Met behulp van de PK-PD benadering is getracht de doseringsvoorschriften van fluoroquinolonen te optimaliseren met als bijkomend doel resistentie inductie bij pathogenen te voorkomen. De effectiviteit van danofloxacine mesylaat en marbofloxacine ten aanzien van een *E. coli* O78/K80 stam werd vergeleken met het doel het selectieve en correcte gebruik van deze antibiotica te bevorderen. De resultaten laten zien dat de farmacokinetische eigenschappen van danofloxacine mesylaat en marbofloxacine, na i.v. toediening, bij kalkoenen overeenkomstig zijn met die bij kippen. Na orale toediening worden beide fluoroquinolonen langzaam geresorbeerd, waarbij de biologische beschikbaarheid bij kalkoenen beter is dan die bij kippen.

In vitro en *in vivo* effectiviteitsonderzoek ten aanzien van *E. coli* O78/K80 toonde aan dat marbofloxacine minder potent is dan danofloxacine mesylaat. In de aanwezigheid van serum vertoonden beide antibiotica een verminderde activiteit. Dit verklaart mogelijk de verschillen in groeisnelheid van pathogenen in cultuurmedia met verschillende samenstelling. Deze gegevens zijn van belang bij vergelijkend onderzoek naar de gevoeligheid van veldisolaten van *E.coli*. De resultaten laten verder zien dat beide fluoroquinolonen concentratieafhankelijke *E. coli* doden; danofloxacine elimineert bacteriën mogelijk bij een lagere PK-PD waarde dan marbofloxacine. Anderzijds heeft

marbofloxacine gunstigere farmacokinetische eigenschappen in de vorm van een lagere plasmaeiwitbinding en een lagere biologische halfwaardetijd. Deze resultaten laten zien dat beide fluoroquinolonen een goede curatieve keuze kunnen zijn bij *E.coli* infecties. Gebaseerd op een PK-PD benadering lijkt marbofloxacine in een orale dagelijkse dosering van 3.0-12.0 mg per kg lichaamsgewicht voor kalkoenen optimaal zijn; voor danofloxacine mesylaat is dat dagelijks 7.7-32 mg per kg. lichaamsgewicht. Beperkingen bij deze benadering zijn echter de verschillende intrinsieke gevoeligheid van veld isolaten, de immuunstatus van de dieren, de pathofysiologische veranderingen als gevolg van de infectie, en de weefselpenetratie van fluoroquinolonen (het PK-PD model gaat immers uit van farmaconconcentraties in bloedserum). Klinische effectiviteitstudies met de voorgestelde doseringsvoorschriften zijn daarom nodig om bacteriële genezing te bevestigen en de afwezigheid van resistentieïnductie ten aanzien van deze antibiotica. Tenslotte, de mogelijkheid bij kalkoenen weefselkamertjes subcutaan te implanteren, maakt het mogelijk in verdere onderzoeken ook de weefselconcentraties door analyse van het weefselvloeistof te bepalen. .

ABC transporters bij pluimvee

MDR1 (P-glycoproteine), MRP2 en BCRP zijn excreterende transmembrane proteïnen, die een belangrijke rol kunnen spelen in de farmacokinetiek van veel geneesmiddel (en toxische stoffen). Bij zoogdieren zijn er toenemende aanwijzingen dat fluoroquinolonen zowel substraat als remmer van ABC transmembrane proteïnen kunnen zijn. Extrapolatie van gegevens van een diersoort naar een andere diersoort is in deze zeer beperkt, omdat er speciesverschillen bestaan in de niveaus van expressie en functionele activiteit van deze transporteiwitten. In dit proefschrift werd daarom de species- en weefselspecifike expressie P-gp, MRP2 en BCRP door middel van nieuw ontwikkelde PCR methoden bepaald; tevens werd de invloed van fluoroquinolonen of expressie van deze transporteiwittenbij bij gezonde en geïnfecteerde dieren onderzocht.

Vergelijkende aspecten bij kippen en kalkoenen

MDR1, MRP2 en BCRP konden aangetoond worden in diverse organen van pluimvee. In het maagdarmkanaal nam het mRNA van MDR1 (P-gp) toe van de maag in de richting van het ileum en opnieuw in het colon van zowel de kip als de kalkoen. Bij zoogdieren zijn soortgelijke patronen bekend. MRP2 mRNA expressie niveaus waren hoger in de dunne darm van beide species; de expressie van BCRP mRNA was meer gelijkmatig in het gehele darmkanaal en in sommige gedeelten, zelfs hoger dan de expressie van MDR1 mRNA. In pluimveelevers was de expressie van MDR mRNA lager dan in het maagdarmkanaal. Dit is ook bij zoogdieren bekend. De mRNA niveaus van MRP2 en

BCRP waren in de lever hoger dan die van MDR1. Deze transporters spelen een rol bij de excretie in geval van lichaamseigen- en lichaamsvreemde stoffen. Net als bij zoogdieren waren de mRNA expressie niveaus van MDR1 en BCRP in de nieren matig tot hoog bij zowel de kip als de kalkoen. Lage gehalten van MDR1 mRNA en hogere gehalten van BCRP mRNA werden gevonden in de bijnieren, longen, hersenen, eierstokken en testikels van kuikens.In de hersenen en bijnieren van kippen kon geen MRP2 mRNA aangetoond worden in tegenstelling tot kalkoenen, waarbij detecteerbare niveaus werden gevonden. Samenvattend, ABC transporters zoals MDR1, MRP2 en BCRP spelen bij vogels net als bij zoogdieren een rol als excretiesystemen voor fysiologische metabolieten, farmaca en hun conjugaten en toxinen en ander toxische stoffen.

Invloed van fluoroquinolonen op het expressieniveau van ABC transport-eiwitten bij gezonde en geïnfecteerde dieren

De orale toediening van danofloxacine mesylaat veroorzaakte een niet significante toename van alle onderzochte genen in de gezonde weefsels van kalkoenen. Dit leidde echter niet tot veranderde plasmaspiegels van fluoroquinolonen, en beïnvloedde ook andere farmacokinetische parameters nauwelijks. De kans op geneesmiddelinteracties is dus gering.

Er zijn aanwijzingen dat onder de factoren die de veranderde expressie van ABC transporter eiwitten veroorzaken, cytokinen afkomstig van ontstekingsreacties een centrale rol vervullen. Twee, in de veterinaire praktijk veel gebruikte fluoroquinolonen herstellen reeds gedurende de therapeutische toepassing de MDR1 mRNA expressie tot een niveau vrijwel gelijk aan dat bij gezonde kippen. Dit ondersteunt de antibacteriële effectiviteit van fluoroquinolonen bij door Gramnegatieve bacteriën veroorzaakte infecties, waardoor minder endotoxinen en vervolgens ook minder proïnflammatoire cytokinen vrijkomen. Deze gegevens vormen een aanwijzing dat tijdens een effectieve behandeling de expressie van MDR1 mRNA hersteld wordt en vervolgens ook het gehalte aan P-glycoproteïne, dat bijdraagt aan de effectiviteit van de darmbarrière.

Fluoroquinolonen als remmers van P-glycoproteïne: een functionele studie

Voor het eerst werd een ex vivo model, gebruikmakend van geïsoleerde splenocyten van kippen, toegepast om de functionele activiteit van ABC transporteiwitten bij deze diersoort te bestuderen. Deze cellen expresseren voornamelijk P-gp, en nauwelijks MRP2 en BCRP. Marbofloxacine-sulfaat, enrofloxacine en ciprofloxacine hadden geen invloed op de functie van deze transporteiwitten. Wel werd enige remming waargenomen na blootstelling aan danofloxacine en danofloxacine mesylaat. Geconcludeerd kan worden dat

de onderzochte fluoroquinolonen mogelijk alleen zwakke remmers zijn van P-gp gemediëerde transportprocessen.

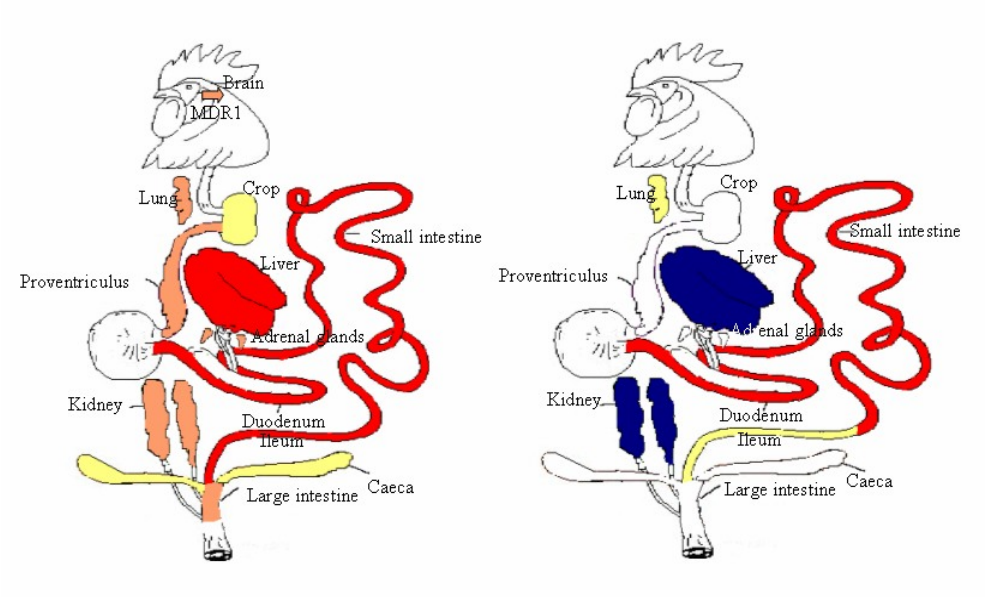
Samenvattende conclusies en toekomstige perspectieven

De resultaten van het in dit proefschrift beschreven onderzoek geven aan dat doseringsvoorschriften van fluoroquinolonen bij pluimvee gebaseerd op een PK-PD benadering belangrijke informaties voor verdere klinische proven verstrekken. Verder kunnen de mogelijkheden, geboden door het gebruik van weefselkamertjes, zoals het meten van extracellulaire antibioticum concentraties in de tijd, de afname van bacteriën na experimentele infectie, en het meten van het optreden van ontstekingsmediatoren leiden tot een verbetering van het PK-PD model en tot een beter inzicht in de farmacokinetiek van fluoroquinolonen.

Het tweede gedeelte van dit proefschrift laat zien dat bij pluimvee een aantal membraan transporteiwitten tot expressie komen, m.n. P-gp, MRP2 en BCRP. De mate van expressie en de functionele activiteit ervan werd enigszins gemoduleerd door fluoroquinolonen, dit in dit onderzoek centraal stonden, en door infecties, b.v. door *E.coli*. De gevonden weefseldistributie van P-gp, MRP2 en BCRP leek op dat beschreven bij zoogdieren, hetgeen aannemelijk maakt dat zij een soortgelijke functie hebben bij de opname, verdeling, biotransformatie en uitscheiding van bepaalde geneesmiddelen en toxische stoffen.

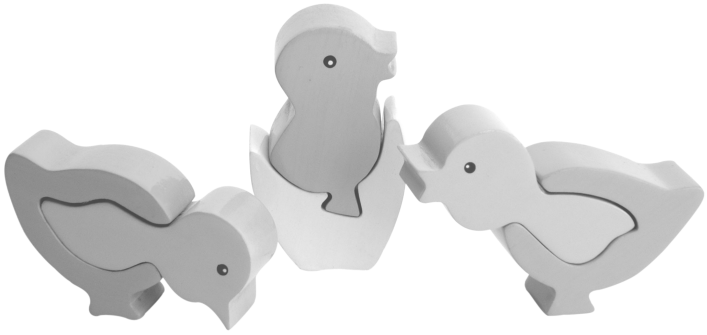
De verschillende functie van deze excreterende transporteiwitten zijn zeer complex. Zij transporteren niet alleen geneesmiddelen (en geneesmiddeleninteracties zijn reeds beschreven bij mensen en laboratoriumdieren) maar tevens talloze endogene stoffen, waardoor zij ook in de pathogenese van ziekteprocessen een rol spelen. De gevonden veranderingen bij geïnfecteerde kippen bevestigen dit. Tevens duiden deze resultaten aan dat meer aandacht besteed moet worden aan de mogelijk invloed van bacteriële en virale infecties, endotoxinen en ontstekingsmediatoren (pro-ïnflammatoire cytokinen) op de expressie en functie van ABC transporters.

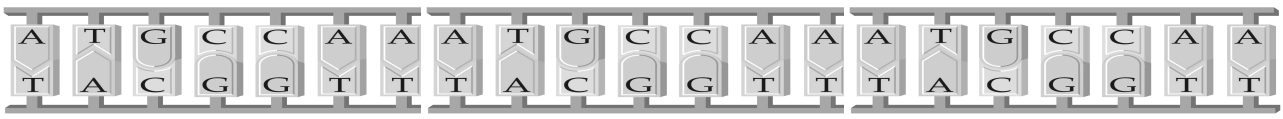
Tenslotte, zal op de mogelijkheid gewezen worden, dat ABC transporters in de toekomst ook belangrijke aangrijpingspunt voor farmaceutische hulpstoffen zijn, met als doel de penetratie van farmaca en vaccins in bepaalde organen (hersenweefsel, testikels, foetale weefsels) te verbeteren.



Figuur: In deze tekening zijn de expressieniveaus van het transporteiwitten P-gp (P-glycoprotein) in organen van het kip weergegeven (een donkere kleur betekend een hoge expressie – en lichte kleur een lage expressie). Dit figuur is afkomstig uit hoofdstuk 5.

РЕЗЮМЕ НА БЪЛГАРСКИ ЕЗИК





Настоящата дисертация разглежда две основни области свързани с терапията на болести при птиците. В първата част се предлагат за валидиране в бъдещи клинични проучвания режими на дозиране на флуорохинолоните, изчислени с помощта на съвременния фармакокинетичен-фармакодинамичен (PK-PD) подход. Той се базира на интегриране на поведението на флуорохинолоните в организма (фармакокинетика) и фармакодинамията им (антимикробната им активност). Като възможност за бъдещо усъвършенстване на фармакокинетичния анализ бе направен опит за подкожно имплантиране на тъканна клетка при пуйки.

Втората част на дисертацията разглежда мембранни транспортни протеини, принадлежащи към семейството на ABC транспортните системи, които пренасят през биологичните мембрани разнообразни субстрати с използване на енергия от хидролиза на АТФ. Те участват в резорбцията, разпределението и елиминирането на лекарствени средства от различни химични групи, включително и флуорохинолони. Увеличават се доказателствата за ролята им във възпалителния процес, както и в патогенезата на значими заболявания. Поради това бяха разработени серия от RT-PCR методи за определяне нивото на ABC транспортните системи в различни органи на пилета и пуйки и за проучване ефекта на флуорохинолоните върху нивата на тези протеини при здрави и болни животни.

Фармакокинетично-фармакодинамично моделиране на флуорохинолони при птици.

Флуорохинолоните са широко използвани за контрол на инфекциозни заболявания в птицевъдството. Въпреки това малко се знае за фармакокинетиката на тези лекарствени средства и възможностите на PK-PD моделиране при определяне на дозовите режими за животински видове с ограничено разпространение, но с икономическо значение като пуйките. Поради това PK-PD моделирането беше приложено като полезен подход в избора на оптимален дозов режим с цел намаляване на риска от селектиране на антимикробна резизсентност. Този метод бе използван и за сравняване на ефикасността на данофлоксацин месилат и марбофлоксацин спрямо патогенен щам *E. coli* O78/K80 според критериите на разумна употреба на антибактериалните средства.

Получените данни показват, че след венозно въвеждане фармакокинетичните характеристики на данофлоксацин месилат и марбофлоксацин при пуйки са сходни с тези при пилета. Двата флуорохинолона се резорбират по-бавно и в по-висока степен при пуйки в сравнение с пилета. Сравняването на *in vitro* и *ex vivo* антимикробната активност на двете лекарствени средства показва, че марбофлоксацина е с по-слаба активност от данофлоксацина спрямо изследвания щам *E. coli* O78/K80. Двата флуорохинолона са с намалена активност в присъствие на серум. Тези данни са в съответствие с други публикувани резултати и най-вероятно отразяват различия в скоростта на бактериалния растеж при разнообразни условия на култивиране.

Настоящото проучване показва, че и двата хинолона имат концентрационно-зависим механизъм на действие спрямо *E. coli* O78/K80 и елиминиране на бактериите при по-ниски стойности на PK-PD параметрита за данофлоксацина. Въпреки това, марбофлоксацина има някои предимства във фармакокинетичните свойства, като ниски степен на свързване с кръвните протеини и скорост на елиминиране, в сравнение с другите флуорохинолони, които би трябвало да компенсират по-слабата му активност спрямо *E. coli* O78/K80. Тези факти показват, че двата флуорохинолона могат да бъдат подходящ избор за постигане на клинично излекуване при инфекции, предизвикани от *E. coli*. Следвайки принципите на PK-PD моделирането и представените данни, могат да се предложат за бъдещо валидиране следните дозови режими: 3.0-12.0 mg/kg/ден за марбофлоксацин и 7.7-32 mg/kg/ден за данофлоксацин. Въпреки редицата предимства, PK-PD моделирането има ясни недостатъци, свързани с невъзможността да се интегрират в математическия модел фактори като различната чуствителност на бактериалните клинични изолати, имунния статус на животните, физиологичните промени по време на възпалителния процес, тъканното разпределение на лекарствените средства, както и факта, че този модел се базира само на серумните или плазмените концентрации. Поради това изчислените дозови режими трябва да бъдат валидирани в клинични опити, при които се проследява не само лечебния ефект, а и селестирането на резистентност спрямо антибактериалните средства. Възможността да се имплантира тъканна клетка при пуйки и да се получава интерстициална течност, паралелно с кръвните проби, предлага по-широки възможности за изучаване кинетиката и фармакодинамичните характеристики на антибиотиците и други лекарствени средства.

ABC транспортни протеини при птици

MDR1 (P-glycoprotein), MRP2 и BCRP са ефлукс трансмембранни протеини, които биха могли да имат съществена роля във фармакокинетиката на голямо разнообразие от лекарствени средства, в лекарствените взаимодействия и в ограничаване резорбцията на токсични вещества. Увеличава се броят на доказателствата за това, че флуорохинолоните са субстрати, нхибитори и модулатори на нивата на ABC транспортните протеини при бозайници. Видовите различия в нивата и функционалната активност на тези протеини ограничават възможностите за междувидово екстраполиране. Поради това настоящото проучване описва нивата на информационната РНК (mRNA) на MDR1, MRP2 и BCRP при птици и изследва влиянието на флуорохинолоните върху тях при здрави и експериментално заразени животни, както и промените във функционалната им активност.

Сравнителни аспекти при пилета и пуйки

MDR1, MRP2 и BCRP бяха установени в различни тъкани на пилета и пуйки. MDR mRNA в стомашно-чревния тракт се увеличава от стомаха до илеума, и отново в колона при двата вида птици, подобно на установеното при бозайници. Нивата на MRP2 mRNA са по-високи в тънките черва при пилета и пуйки, а тези на BCRP са сходни в целия стомашно-чревен тракт на пуйки и по-високи от тези на MDR1. Значително присъствие на MDR1 mRNA в черния дроб (по-ниски нива от тези в червата) е типично за бозайници и птици. В черния дроб на птиците бяха установени по-високи нива на MRP2 и BCRP mRNA, в сравнение с MDR1 mRNA, отразяващи ролята на тези протеини в жлъчната екскреция на ендогенни субстанции и ксенобиотици. Нивата на MDR1 и BCRP mRNA в бъбреците на бозайници и птици са ниски до средни. Ниски нива на MDR1 и високи на BCRP mRNA бяха открити в надбъбречните жлези, белите дробове, мозъка, яичниците и тестисите при полово незрели птици. MRP2 mRNA не беше открита в мозъка и надбъбречните жлези при пилета, но в тези органи при пуйки бяха намерени установими нива.

Имайки предвид филогенетичното родство на ABC транспортните протеини, може да се допусне, че при птиците MDR1, MRP2 и BCRP биха имали същата роля на ефлукс помпи за физиологични субстанции, токсини, лекарства и техните конюгирани метаболити от втората фаза, както при бозайниците.

Ефект на флуорохинолоните върху mRNA нивата на ABC транспортните протеини при здрави и заразени птици

Данофлоксацинът под формата на месилат предизвиква статистически незначимо увеличаване на mRNA нивата на изследваните протеини в тъканите на здрави пуйки. Това увеличаване е по-ясно изразено след последователно вътрешно третиране с флуорохинолона. Тези промени не водят до изменения в серумните нива и фармакокинетичните параметри на вътрешно въведения хинолон. Това е индикация за малък риск от настъпването на клинично значими лекарствени взаимодействия. Увеличащият се брой даказателства подсказват, че сред факторите които модулират нивата на ABC транспортните протеини са възпалителните реакции като водеща роля има освобождаването на различните цитокини. Третирането с данофлоксацин и енрофлоксаци, два хинолона, често прилагани в практиката, води до значимо възстановяване на физиологичните нива на MDR1 mRNA, въпреки че не се достигат тези при здравите птици. Промените, настъпили в резултат на терапията с двата флуорохинолона, по-скоро са следствие от ограничаване на инфекцията, което води до намаляване на негативните въздействия от освобождаването на ендотоксините от Грам-отрицателни микроорганизми (LPS) и в последствие подтискане освобождаването на цитокини. Нивата на MRP2 mRNA, която е с ограничено разпространение в сравнение с MDR1 mRNA, не бяха значимо променени от

инфекцията, но бе установена сходна тенденция на увеличаване в резултат от третирането. Изключение бе статистически значимото намаляване на MRP2 mRNA в черния дроб след третиране с данофлоксацин месилат. Взети заедно, тези данни предполагат, че в резултат от ефективна терапия, постепенното възстановяване на понижените нива на MDR1 mRNA (и следователно P-gp) допринася за ефективното функциониране на интестиналната бариера.

Флуорохинолоните като инхибитори на P-gp: функционално изследване

Функционалната активност на ABC транспортните протеини при пилета бе проучена за първи път с помощта на *ex vivo* модел, базиран на използването на лимфоцити, изолирани от далак. Проведените опити с широкоспектърни и специфични инхибитори на изследваните ефлукс протеини, показват че с този модел може да се характеризира основно функцията на P-gp. Този факт се подкрепя от установените достатъчно високи MDR1 mRNA нива в лимфоцитите. За разлика от P-gp, нивата на MRP2 и BCRP mRNA бяха твърде ниски, което се потвърждава и от резултатите от функционалното изследване с инхибитори, специфични за тези протеини. Нашите данни сочат, че марбофлоксацина, енрофлоксацина и ципрофлоксацина не повлияват ефлукс протеините на функционално ниво. Слаб инхибиращ ефект бе установен при тестването на данофлоксацин и данофлоксацинов месилат. Тези данни позволяват да се обобщи, че проучваните флуорохинолони, които често се използват във ветеринарномедицинската практика, са слаби инхибитори на транспорта, осъществяван с участието на P-gp. Остава открит въпросът за влиянието на тези лекарствени средства върху функцията ня други ефлукс протеини.

Обобщение и бъдещи перспективи

В заключение, данните от настоящото проучване показват, че PK-PD моделирането може да бъде прилагано при сравняване на активността на флуорохинолоните и при определяне на дозови режими за тестване в клинични опити. Валидирането на предложените дозови режими се налага от ясните ограничения на PK-PD подхода. Възможността за използване на тъканни клетки и в последствие за измерване на лекарствената концентрация, броя на бактериалните единици при експериментална инфекция, както и медиаторите на възпаление повишава практическата значимост на този модел, който би могъл да се използва в предстоящи PK и PK-PD експерименти.

Втората част от дисертацията доказва, че ефлукс протеините, кодирани от MDR1, MRP2 и BCRP гените, се установяват при птици и техните нива и функционална активност може да се модулират от представители на флуорохинолоните и експериментална инфекция с *E. coli*. Тъканната локализация на

MDR1, MRP2 и BCRP съответства на описаната при бозайници, което предполага сходна функция в резорбцията, разпределението, метаболизма и екскрецията на лекарствените средства. Бъдещи проучвания на ABC транспортните протеини би трябвало да разкрие вероятните междувидови, породни, полови и възрастови различия в техните нива при птици, които биха имали клинично значение.

Значимостта на участието на проучваните ефлукс протеини в лекарствените взаимодействия и фармакокинетиката на различни субстанции, широко използвани в птицевъдството, все още не е добре проучена. По същия начин стои въпросът с ролята на тези транспортни протеини като модулатори на резорбцията и разпределението и екскрецията (вкл. яйцата) на токсини и други нежелани примеси във фуража. Представеното проучване със заразени пилета подсказва, че повече внимание трябва да се обърне на ефекта на бактериалните ендотоксини, вирусни инфекции и възпаление (вкл. и на освобождаването на медиатори на възпалението като цитокини) върху нивата и функцията на ABC транспортните протеини. Функцията на тези протеини като бариера за проникването на разнообразни вещества в отделни органи може да бъде използвана и с цел подобряване на проникването на лекарствени средства и ваксини до прицелните тъкани.

Curriculum vitae

Aneliya Milanova Haritova was born on August 19th, 1971, in Yambol, Bulgaria. After secondary school she started her studies in veterinary medicine at Faculty of Veterinary Medicine, High Institute of Zootechnics and Veterinary Medicine, now Trakia University in 1989 and graduated in 1995 as veterinarian. She worked as a practitioner up to 1997. She started as a Ph.D. student at the Department of Pharmacology, Toxicology and Therapeutics in February 1998 under supervision of Prof. D. Pashov. The project related to investigation of pharmacokinetics of antibacterial drugs in animal species of veterinary interest was finalized with defense of Ph.D. thesis in 2001. Since 2002 she started as an assistant professor at the Department of Pharmacology, Faculty of Veterinary Medicine, Trakia University. Professional training included also courses on PK-PD approach and pharmacokinetics, organized by ECVPT. After successful application in 2003 she received a grant from NWO, The Netherlands and NSFB, Ministry of Education and Science, Bulgaria for implementation of a one year project during 2004, related to PK-PD modeling of fluoroquinolones in poultry. The work on the project was extended with investigations of ABC transporters in poultry and it develops into a Ph.D. project under supervision of Prof. J. Fink-Gremmels at the Department of Veterinary Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, Utrecht University. Part of the experimental work was performed at the Department of Pharmacology, Veterinary Physiology and Physiological Chemistry, Faculty of Veterinary Medicine, Trakia University, Bulgaria under the supervision of Prof. L. Lashev.

List of Publications

Haritova, A., N. Rusenova, P. Parvanov, L. Lashev and J. Fink-Gremmels, 2006. Pharmacokinetic-Pharmacodynamic modeling of danofloxacin in turkeys. Veterinary Research Communications, (in press)

Haritova, A., N. Rusenova, P. Parvanov, L. Lashev and J. Fink-Gremmels, 2006. Integration of pharmacokinetic and pharmacodynamic indices of marbofloxacin in turkeys. Accepted in Antimicrobial Agents and Chemotherapy

Haritova, A., J. Schrickx, L. Lashev and J. Fink-Gremmels, 2006. ABC efflux transporters – the 3rd dimension in kinetics not only of fluoroquinolones. Submitted to Bulgarian Journal of Veterinary Medicine

Haritova, A. and H. Hubenov, Implantation of Tissue Chambers in Turkeys: A Pilot Study. Submitted to Bulgarian Journal of Veterinary Medicine

Haritova, A., J. Schrickx and J. Fink-Gremmels, Expression of Drug Efflux Transporters in Poultry Tissues. Submitted to Poultry Science

Haritova, A., J. Schrickx and J. Fink-Gremmels, Functional Studies on the Activity of Efflux Transporters in an Ex Vivo Model with Chicken Splenocytes and Evaluation of Selected Fluoroquinolones in This Model. Submitted to Biochemical Pharmacology

Abstracts

Haritova, A., J. Schrickx and J. Fink-Gremmels, Effect of fluoroquinolones on P-glycoprotein activity in chickens. 10th International Congress of the European Association for Veterinary Pharmacology and Toxicology, Torino, Italy, September, 2006

Haritova, A., N. Rusenova, A. Rusenov, J. Schrickx, L. Lashev and J. Fink-Gremmels, Effect of fluoroquinolone treatment on MDR1 and MRP2 expression in chickens with experimental *E. coli* infections. 10th International Congress of the European Association for Veterinary Pharmacology and Toxicology, Torino, Italy, September, 2006

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