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Comparative activity of pradofloxacin and marbofloxacin against coagulase-positive staphylococci in a pharmacokinetic–pharmacodynamic model based on canine pharmacokinetics

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Pradofloxacin (PRA), a novel veterinary 8-cyano-fluoroquinolone (FQ), is active against *Staphylococcus pseudintermedius*, the primary cause of canine pyoderma. An *in vitro* pharmacokinetic–pharmacodynamic model was used to compare the activities of PRA and marbofloxacin (MAR) against three clinical isolates of *S. pseudintermedius* and reference strain *Staphylococcus aureus* ATCC 6538. Experiments were performed involving populations of 10^{10} CFU corresponding to an inoculum density of approximately 5×10^7 CFU/mL. The time course of free drug concentrations in canine serum was modelled, resulting from once daily standard oral dosing of 3 mg of PRA/kg and 2 mg of MAR/kg. In addition, experimentally high doses of 6 mg of PRA/kg and 16 mg of MAR/kg were tested against the least susceptible strain. Viable counts were monitored over 24 h. At concentrations associated with standard doses, PRA caused a faster and more sustained killing than MAR of all strains. The ratios of free drug under the concentration–time curve for 24 h over MIC and the maximum concentration of free drug over MIC were at least 90 and 26, and 8.5 and 2.1 for PRA and MAR, respectively. At experimentally high doses, PRA was superior to MAR in terms of immediate killing. Subpopulations with reduced susceptibility to either FQ did not emerge. We conclude that PRA is likely to be an efficacious therapy of canine staphylococcal infections.

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INTRODUCTION

Pyoderma is a common bacterial skin infection in dogs (Scott & Paradis, 1990; Lund *et al.*, 1999). It can be divided into superficial and deep pyoderma (Scott *et al.*, 2001). Deep pyoderma, which is often associated with cellulitis, can be difficult to treat because bacteria in lesions are often in the centre of inflammatory foci (Mueller & Stephan, 2007). Pyoderma is mainly caused by Gram-positive bacteria (most notably coagulase-positive and coagulase-negative staphylococci) but also by Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli* (Mueller & Stephan, 2007). The most important pathogen associated with canine pyoderma is *Staphylococcus pseudintermedius*, a resident of the normal skin (Fitzgerald, 2009). Treatment for both pyoderma forms involves antibiotic therapy in which lesions associated with deep pyoderma require long-term oral antibiotic therapy (DeBoer, 1990). Established fluoroquinolones (FQ) have been considered to be appropriate drugs for treatment for canine pyoderma owing to their favourable pharmacokinetic and pharmacodynamic properties (Horspool *et al.*, 2004). However, successful outcome of treatment has been threatened by the emergence of FQ resistance among staphylococcal isolates (Morris *et al.*, 2006).

Pradofloxacin (PRA) is a new 8-cyano-FQ recently approved in the European Union for the treatment for the following infections caused by susceptible strains of the *Staphylococcus intermedius* group (including *S. pseudintermedius*) and other specified susceptible bacteria: superficial and deep pyoderma, wound infections, acute urinary tract infections and as adjunctive treatment for severe gingival and periodontal tissue infections in dogs, and

against wound infections, abscesses and acute upper respiratory tract infections in cats (Stephan *et al.*, 2003, 2006a,b; Mueller & Stephan, 2007; Spindel *et al.*, 2008; Restrepo *et al.*, 2010). The drug exhibits *in vitro* activity against a broad spectrum of aerobic and anaerobic Gram-positive and Gram-negative bacteria, including *S. pseudintermedius* (Abraham *et al.*, 2002; Silley *et al.*, 2007). Like other FQs, PRA acts by a concentration-dependent killing mechanism and exerts postantibiotic and postantibiotic sub-MIC effects against FQ-susceptible Gram-positive and Gram-negative pathogens (Wetzstein, 2008). In contrast to enrofloxacin and marbofloxacin (MAR), PRA has been suggested to display activity against stationary phase and nondividing cells of *S. intermedius* and *Staphylococcus aureus*, including variants with reduced susceptibility to FQs (Koerber *et al.*, 2002).

In vitro pharmakcokinetic–pharmacodynamic (PK-PD) models represent a useful tool to model concentration–time curves of a drug as achieved *in vivo*, thus allowing characterization of the relationship between drug concentration and antibacterial activity. Furthermore, PD parameters can be used to calculate PK-PD indices such as the ratio of the peak drug concentration over MIC (C_{max}/MIC), the time of the drug concentration above the MIC (T>MIC) and the ratio of the area under the concentration–time curve between time zero (t_0) and 24 h over MIC ($AUC_{0–24\ h}$/MIC). Previous reports suggest that C_{max}/MIC and $AUC_{0\text{-}24\ h}$/MIC are the most important PK-PD indices predicting the efficacy of FQs (Zinner & Firsov, 1999; Craig, 2001; Zelenitsky *et al.*, 2003). $AUC_{0\text{-}24\ h}$/MIC ratios of >100–125 and C_{max}/MIC ratios of >8–10 have been recommended to predict high clinical and microbiological efficacy as well as to limit the development of bacterial resistance for concentration-dependent drugs, although FQs have been shown to maintain activity against Gram-postive bacteria at lower $AUC_{24\ h}$/MIC ratios than for Gram-negative bacteria (McKellar *et al.*, 2004). In order to assess the AUC/MIC or C_{max}/MIC ratio accurately, serum drug concentrations need to be corrected in order to reflect the extent of protein binding (Wright *et al.*, 2000).

The aim of the present study was to compare the antibacterial effects of PRA and MAR against three clinical isolates of *S. pseudintermedius* and a reference strain of *S. aureus* in an one-compartment *in vitro* PK-PD model, based on canine PK. Inocula of 10^{10} CFU (approximately 5×10^7 CFU/mL) were exposed to free drug (protein unbound; *f*) concentrations resulting from once daily standard oral dosing of 3 mg of PRA/kg and 2 mg of MAR/kg. In addition, experimentally high doses of 6 mg of PRA/kg and 16 mg of MAR/kg were tested against the least susceptible *S. pseudintermedius* strain in order to safely achieve drug concentrations facilitating comparably high $fAUC_{0\text{-}24\ h}$/MIC and fC_{max}/MIC ratios of >125 and >10, respectively, which are most frequently quoted in the literature.

MATERIALS AND METHODS

Bacterial strains

Tested strains included three clonally unrelated clinical isolates of *S. pseudintermedius* (41JD002, 65MD004, 116AD022), recovered from dogs as well as a reference strain, *S. aureus* ATCC 6538. Isolates were identified employing the BBL Crystal™ GP ID Kit (Becton Dickinson & Co., Sparks, MD, USA), Bactident® coagulase (Merck, Darmstadt, Germany), β-hemolysis on Columbia agar supplemented with 5% defibrinated sheep blood, lack of yellow pigmentation (typical of *S. aureus*) and a negative Slidex® Staph plus reaction (bioMérieux® SA, Marcy-I'Etoile, France). None of the isolates carried a point mutation in the quinolone resistance-determining region of *grlA*, the gene encoding subunit A of topoisomerase IV (P. Heisig, University of Hamburg, personal communication). The strains were stored as permanent cultures at −80 °C. Thawn stocks were subcultured on Mueller-Hinton agar (Becton Dickinson & Co.) plates with 5% sheep blood (elocin-lab, Gladbeck, Germany). Identification as *S. pseudintermedius* was confirmed by PCR-restriction fragment length polymorphism (RFLP) analysis recently described by Bannoehr *et al.* (2009).

Antimicrobial agents

PRA (lot HMT 4273-99) and MAR (lot HLR 3697) were provided by Bayer Animal Health GmbH, Leverkusen, Germany. Chemical purities of the drugs have been described elsewhere (Wetzstein, 2005).

Susceptibility testing

MICs were determined by the broth microdilution procedure with twofold serial dilutions in Mueller-Hinton II broth (Becton Dickinson & Co.) with an inoculum of approximately 5×10^5 CFU/mL, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). All MICs were determined at least fourfold.

In vitro pharmacokinetic–pharmacodynamic model

The model described by Grasso *et al.* (1978) was used with slight modifications (Keil & Wiedemann, 1995). A schematic representation of the apparatus is presented in Fig. 1. The model simulates the PK one-compartment open model with first-order absorption, as observed in serum after oral administration of the drug, and comprised three flasks that were connected with a Tygon® tube system (Ismatec, Wertheim-Mondfeld, Germany) and a peristaltic pump. Flask A, containing the antibiotic solution at volume V_A, was connected with flask C containing the bacterial culture of the test strain. Flask C was kept at 37 °C and had a constant volume V_C of broth (Table 1). Homogeneity of the bacteria was ensured by a magnetic stirrer. Flask R (reservoir) contained drug-free medium. To model drug absorption and elimination, the peristaltic pump transferred either antibiotic solution or drug-free medium into flask C.

Overnight cultures of the test organisms were diluted 1:5 into fresh Mueller-Hinton II broth. The suspensions were incubated at 37 °C for 1.5 h to achieve an initial inoculum density of approximately 5×10^7 CFU/mL in flask C (216 mL), thus providing for populations of 10^{10} CFU. Strains were exposed to free drug concentrations of PRA or MAR resulting from once

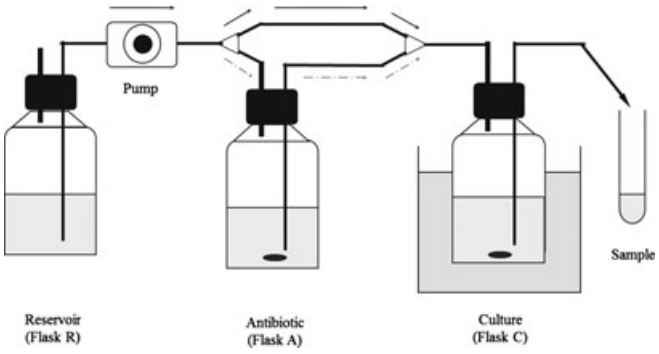

Fig. 1. Model of the apparatus for simulation of the time curves of antibiotic concentrations. Dashed grey arrows indicate the flow direction until maximum concentration (C_{max}) was achieved in flask C. The flow diection was changed after C_{max} had been achieved (black arrows). Flasks A and C were protected from light.

daily standard oral dosing of 3 mg of PRA per kg bw and of 2 mg of MAR per kg bw in canine serum. In addition, experimentally high doses of 6 mg of PRA per kg bw or 16 mg of MAR per kg bw were applied to evaluate the activity of both drugs against the least susceptible *S. pseudintermedius* strain. Flasks containing the antibiotic were protected from light. Samples of 500 μL were taken at 0, 1, 2, 4, 6, 8, 12 and 24 h from flask C for the determination of FQ concentrations and colony counting.

Pharmacokinetics

The PK data of PRA and MAR were obtained from studies in healthy beagles performed by the study groups of Cester *et al.* (1996) and Fraatz *et al.* (2002), respectively, and then re-evaluted. The plasma PK of MAR was assessed in a cross-over study comparing MAR with enrofloxacin. Eight healthy adult dogs were used. Plasma samples were analyzed by a microbiological assay as well as by HPLC. HPLC data were employed. Serum pharmacokinetics of PRA were evaluated in a dose-ranging study with four parallel groups of eight healthy adult dogs each. PRA concentrations had been determined by turbulent flow chromatography/tandem mass spectrometry (TFC-MS/MS). Pharmacokinetic parameters were calculated by compartmental analysis using nonlinear least square regression analysis and a one-compartment model after oral application using the software programme GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA). One-compartment model analysis resulted in best fit of the data as judged by minimal absolute sum of squared residuals, indicating a mean residual of 0.9% with respect to Cmax values.

Data on protein binding of the study drugs were taken from investigations performed by Bregante *et al.* (Bregante *et al.*, 2000, 2003). Pharmacokinetic parameters are presented in Table 1. The flow rate (Fα) to model drug absorption as well as the volumes of flasks A and C were calculated using the software programme CompaKom. The flow rate to model drug elimination was calculated by multiplying the volume in flask

Table 1. Pharmacokinetic parameters describing drug concentration–time curves associated with once daily oral dosing of PRA and MAR and parameters applied to the *in vitro* model for simulation

			PK parameters							*In vitro* model parameters							
			C_{max} (mg/L)				$AUC_{0\text{-}24\ h}$ (mg × h/L)		Rate constant (h^{-1})	Rate constant (h^{-1})		Flask					
												A			*C*		*R*
FQ	Dose (mg/kg)	Protein binding (%)	Total drug[a]	Free drug[b]	T_{max}[c] (h)	$t_{1/2}$[d] (h)	Total drug[e]	Free drug[f]	Ka^g	Ke^g	Ka^h	Ke^h	V_A (mL)	C^i (mg/L)	V_C (mL)	V_R (mL)	
Standard dose																	
PRA	3	36	1.66	1.06	1.6	7.0	17.7	11.3	2.04	0.10	1.18	0.09	50.6	7.25	216.4	800	
MAR	2	25	1.37	1.03	1.9	9.3	17.5	13.1	1.74	0.07	0.98	0.07	61.4	6.09	216.4	600	
Experimental dose																	
PRA	6	36	3.33	2.13	1.6	6.9	35.2	22.5	1.99	0.10	1.18	0.09	50.6	14.5	216.4	800	
MAR	16	25	10.97	8.23	1.9	9.3	140.1	105.1	1.75	0.07	0.98	0.07	61.4	48.7	216.4	600	

PK profiles for PRA and MAR were derived from Fraatz *et al.* (2002) and Cester *et al.* (1996). Data on protein binding were taken from the group of Bregante (Bregante *et al.*, 2000, 2003).
[a]Maximum concentration of total drug in serum.
[b]Maximum concentration of free drug in serum.
[c]Time to maximum concentration in serum.
[d]Half-life.
[e]Area under the total drug concentration–time curve (0–24 h).
[f]Area under the free drug (protein unbound) concentration–time curve (0–24 h).
[g]Rate constant for the absorption (Ka) and the elimination (Ke) phase as evaluated from compartimental analysis.
[h]Rate constant for the absorption (Ka) and the elimination (Ke) phase as applied to the *in vitro* model.
[i]Concentration of FQ in flask A; V_A, Volume of flask A; V_C, Volume of flask C; V_R, Volume of flask R.

C by the elimination rate constant Ke. The absorption rate constant Ka and the elimination rate constant Ke were calculated according to the equations Ka (h^{-1}) = Fα (mL/h)/V_A (mL) and Ke (h^{-1}) = ln C_1 − ln $C_2/t_2 - t_1$ (h), where C_1 is the concentration at time 1 (t_1) and C_2 is the concentration at time 2 (t_2).

Microbiological assay for determining FQ concentrations

Concentrations of PRA or MAR in flask C were determined by an agar diffusion assay using Iso-sensitest agar (Oxoid, Wesel, Germany) and the FQ-susceptible strain *Klebsiella pneumoniae* CR1 as indicator organism. Antibiotic standards were prepared in Iso-sensitest broth (Oxoid, Wesel, Germany). Cavities (diameter = 9 mm) in agar plates, which had been preswabbed with approximately 10^6 CFU/mL, were filled with 100 μL of either a FQ quality control concentration or a sample collected from flask C. Quality control concentrations were 0.025, 0.05, 0.1 and 0.2 mg/L for PRA and 0.05, 0.1, 0.2 and 0.4 mg/L for MAR. Samples from the model expected to contain higher concentrations than 0.2 and 0.4 mg/L were diluted 1:5 up to 1:40 to achieve concentrations within the respective range. All samples were tested in triplicate. Agar plates were incubated at 37 °C for 18 h. Antibiotic concentrations were calculated by comparing the zone diameters of growth inhibition obtained for samples from the model with those obtained for the quality control samples. Bioassays were found to be linear over the ranges of quality control concentrations ($r > 0.98$). Between-day coefficients of variation (CV%) for assays of quality control concentrations ranged from 0% to 6.5% for PRA and 0% to 10.7% for MAR.

Antibacterial effects and time-kill data

Viable counts were determined by plating 50 μL aliquots of serial 10-fold dilutions onto Tryptic Soy agar plates (Fluka, Buchs, Switzerland). Colonies were enumerated after 48 h of incubation at 37 °C. The lower limit of detection was one colony per plate (20 CFU/mL). Viable counts were corrected for the loss of bacteria owing to dilution during the model experiments, as described previously (Keil & Wiedemann, 1995). All experiments were performed at least in duplicate. Time-kill curves were plotted as $\log_{10}$ CFU per mL over time.

Detection of variants with reduced FQ susceptibility

Two samples of 100 μL each, taken at 0, 6, 12 and 24 h, were spread onto Tryptic Soy agar plates containing PRA or MAR at concentrations ranging from 0.25 to four times the MIC. Plates were incubated at 37 °C for up to 7 days and checked daily for bacterial growth.

MPC testing

Mutant prevention concentrations (MPCs) were determined as described previously (Wetzstein, 2005). In brief, BioAssay dishes with a culture area of 530 cm^2 (Nalgene Nunc International Corp., Rochester, NY, USA) were filled with 150 mL of Difco Balanced Sensitivity Test medium pH 7.4 ± 0.2 (Becton Dickinson & Co.) containing a specific FQ concentration. Aliquots of 0.8 mL of a cell suspension containing approximately 5×10^9 CFU were then streaked onto each plate. Plates were incubated in sealed plastic bags in the dark at 36 ± 1 °C for up to 14 days. Viable counts were determined and plotted over the respective FQ concentration. All experiments were performed at least in triplicate. The MPC indicates the lowest concentration that prevents growth of the least susceptible first-step variants contained in the bacterial population employed (Drlica & Malik, 2003).

To evaluate PK-PD indices involving MPCs and prevention of the emergence of resistant mutants, fC_{max}/MPC and $fAUC_{0\text{-}24\ h}$/MPC ratios were calculated for the standard and experimental doses of PRA and MAR.

Statistical analyses

The statistical analyses were performed using the software GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA). The parameters for PRA and MAR were compared using a two-factor analysis of variance (ANOVA). The factors were time (repeated) and drug. A value of $P < 0.05$ was considered as significant.

RESULTS

MICs and MPCs for the test organisms

MICs of PRA and MAR ranged from 0.031 to 0.125 mg/L and from 0.125 to 0.5 mg/L, respectively. Mean MPCs of MAR were sixfold higher than those of PRA for *S. aureus* ATCC 6538 and two *S. pseudintermedius* strains (41JD002 and 65MD004), and tenfold higher for *S. pseudintermedius* 116AD002 (Table 2).

Pharmacokinetic modelling

Drug concentrations measured by the agar diffusion assay during each of the model experiments closely matched the target free drug concentration–time profiles, with the exception that concentrations of MAR simulating high experimental dosing were slightly below the target concentration–time curve (Fig. 2).

Time-kill kinetics at simulated concentration–time curves resulting from standard dosing

PRA caused a more rapid and sustained killing than MAR against all strains. Against *S. aureus* ATCC 6538, PRA achieved a 2 $\log_{10}$ reduction at 6 h, followed by an increase of 0.4 $\log_{10}$ after 12 h. MAR was substantially less active than PRA, with a decrease of <0.5 $\log_{10}$ at 6 h and subsequent re-growth (Fig. 3a). In experiments with *S. pseudintermedius* 41JD002, a 3 $\log_{10}$ reduction in viable counts was achieved by PRA at 6 h, increasing to a 4 $\log_{10}$ reduction at 12 h (Fig. 3b). Against

Table 2. Pharmakcokinetic–pharmacodynamic (PK-PD) ratios for PRA and MAR

		MIC-based PK-PD data			MPC-based PK-PD data	
Bacterial strain	Parameter	PRA	MAR	Parameter	PRA	MAR
Staphylococcus intermedius 41JD002	MIC (mg/L)	0.0625	0.25	MPC_{Range} (mg/L)	0.25–0.3	1.5–1.75
				MPC_{Mean} (mg/L)	0.28	1.63
	Standard dose					
	C_{max}/MIC	26.6	5.5	C_{max}/MPC[a]	5.9	0.84
	fC_{max}/MIC	17.0	4.1	fC_{max}/MPC[a]	3.8	0.63
	$AUC_{0\text{-}24\ h}$/MIC (h)	283	70	$AUC_{0\text{-}24\ h}$/MPC (h)[a]	63.2	10.7
	$fAUC_{0\text{-}24\ h}$/MIC (h)	180	52	$fAUC_{0\text{-}24\ h}$/MPC (h)[a]	40.4	8.0
S. intermedius 65MD004	MIC (mg/L)	0.0625	0.25	MPC_{Range} (mg/L)	0.25–0.3	1.5–1.75
				MPC_{Mean} (mg/L)	0.28	1.63
	Standard dose					
	C_{max}/MIC	26.6	5.5	C_{max}/MPC[a]	5.9	0.84
	fC_{max}/MIC	17.0	4.1	fC_{max}/MPC[a]	3.8	0.63
	$AUC_{0\text{-}24\ h}$/MIC (h)	283	70	$AUC_{0\text{-}24\ h}$/MPC (h)[a]	63.2	10.7
	$fAUC_{0\text{-}24\ h}$/MIC (h)	180	52	$fAUC_{0\text{-}24\ h}$/MPC (h)[a]	40.4	8.0
S. intermedius 116AD022	MIC (mg/L)	0.125	0.5	MPC_{Range} (mg/L)	0.25–0.3	2.75–3.0
				MPC_{Mean}(mg/L)	0.28	2.88
	Standard dose					
	C_{max}/MIC	13.3	2.7	C_{max}/MPC[a]	5.9	0.48
	fC_{max}/MIC	8.5	2.1	fC_{max}/MPC[a]	3.8	0.36
	$AUC_{0\text{-}24\ h}$/MIC (h)	142	35	$AUC_{0\text{-}24\ h}$/MPC (h)[a]	63.2	6.1
	$fAUC_{0\text{-}24\ h}$/MIC (h)	90	26	$fAUC_{0\text{-}24\ h}$/MPC (h)[a]	40.4	4.6
	Experimental dose					
	C_{max}/MIC	26.6	21.9	C_{max}/MPC[a]	11.9	3.8
	fC_{max}/MIC	17.0	16.5	fC_{max}/MPC[a]	7.6	2.9
	$AUC_{0\text{-}24\ h}$/MIC (h)	282	280	$AUC_{0\text{-}24\ h}$/MPC (h)[a]	126	48.6
	$fAUC_{0\text{-}24\ h}$/MIC (h)	180	210	$fAUC_{0\text{-}24\ h}$/MPC (h)[a]	80.4	36.5
S. aureus ATCC 6538	MIC (mg/L)	0.03125	0.125	MPC_{Range} (mg/L)[b]	0.5–0.6	3–3.5
				MPC_{Mean} (mg/L)[b]	0.55	3.25
	Standard dose					
	C_{max}/MIC	53.1	11.0	C_{max}/MPC[a]	3.0	0.42
	fC_{max}/MIC	33.9	8.2	fC_{max}/MPC[a]	1.9	0.32
	$AUC_{0\text{-}24\ h}$/MIC (h)	566	140	$AUC_{0\text{-}24\ h}$/MPC (h)[a]	32.2	5.4
	$fAUC_{0\text{-}24\ h}$/MIC (h)	362	105	$fAUC_{0\text{-}24\ h}$/MPC (h)[a]	20.5	4.0

[a]Mean Mutant prevention concentrations (MPC) values were employed.
[b]Data from Wetzstein, 2005.

S. pseudintermedius strains 65MD004 and 116AD022, PRA achieved a 2 log_{10} reduction at 6 h, followed by a further decrease until 12 h (Fig. 3c,d). Thereafter, viable counts of all three strains increased slightly, but remained 2–3.7 log_{10} below the starting inoculum. In contrast, MAR was bacteriostatic against all strains (<1 log_{10} killing) at 6 h. At 24 h, a decrease of 1.9 and 1 log_{10} in viable counts was achieved for 41JD002 and 65MD004, respectively, while re-growth was observed for 116AD022 after 8 h, with viable counts above the initial inoculum at 24 h.

Time-kill kinetics at simulated concentration–time curves resulting from experimentally high dosing

Experiments were performed with *S. pseudintermedius* 116AD022, as this strain displayed the lowest susceptibility to PRA (MIC = 0.125 mg/L) and MAR (MIC = 0.5 mg/L). Experimental doses of PRA (6 mg/kg) and MAR (16 mg/kg) resulted in ratios of $fAUC_{0\text{-}24\ h}$/MIC and fC_{max}/MIC that were comparably high for both drugs: 180 and 17.0, respectively, for PRA and 210 and 16.5, respectively, for MAR (Table 2).

When applied at high experimental doses, both drugs caused ≥3 log_{10} reductions in viable counts at 12 h, but PRA still displayed a more rapid killing effect within the first 8 h, as compared to MAR (Fig. 3e). After 12 h, with MAR viable counts decreased further, while PRA allowed for an increase of 0.5 log_{10}.

Occurrence of mutants with reduced FQ susceptibility

Subpopulations with reduced susceptibility to either drug were not detected. The MICs of PRA and MAR remained unchanged for all strains during any of the experiments.

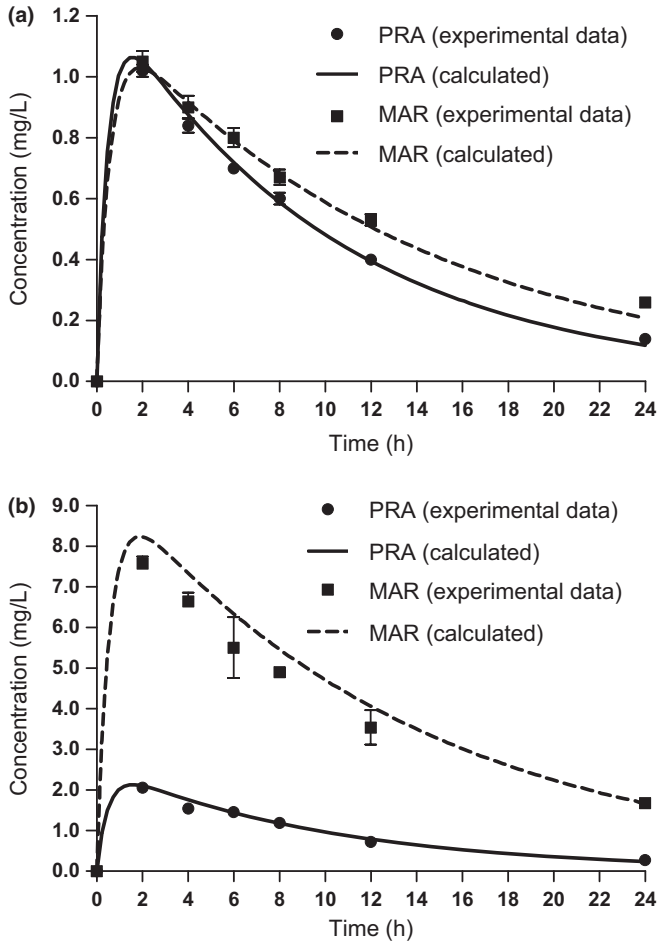

Fig. 2. Comparison of the calculated canine free drug concentration–time curves and experimental data associated with once daily oral dosing of 3 mg PRA/kg or 2 mg MAR/kg (standard dosing, panel a; graphs are means of at least nine experiments) and 6 mg PRA/kg or 16 mg MAR/kg (experimentally high dosing, panel b; graphs are means of at least two experiments). Bars represent standard deviations.

MIC and MPC based PK-PD ratios

Data are presented in Table 2. Standard doses of PRA resulted in ratios of $fAUC_{0\text{-}24h}$/MIC and fC_{max}/MIC that were at least three times higher than those of MAR. fC_{max}/MPC and $fAUC_{0\text{-}24\ h}$/MPC ratios associated with the standard doses were 1.9–3.8 and 20.5–40.4, respectively, for PRA and 0.32–0.63 and 4.0–8.0, respectively, for MAR. Those for the experimentally high doses were 7.6 and 80.4, respectively, for PRA and 2.9 and 36.5, respectively, for MAR.

DISCUSSION

The aim of the present study was to compare the antibacterial activity of PRA and MAR against *S. pseudintermedius* and *S. aureus* in an PK-PD model. Large bacterial populations of 10^{10} CFU (inocula of approximately 5×10^7 CFU/mL in 216 mL) were used to detect possible mutants with reduced susceptibility to the study drugs. Free drug steady-state concentrations observed in the serum of healthy beagle dogs, associated with the oral administration of standard therapeutic dosages of PRA (3 mg/kg once daily) and MAR (2 mg/kg once daily), were simulated (Cester *et al.*, 1996; Fraatz *et al.*, 2002). Moreover, the killing effects exerted by experimentally high doses were investigated with the least susceptible strain. As the extent of protein binding of FQs varies with the method employed, we applied 36% for PRA and 25% for MAR, as determined by Bregante and collaborators (Bregante *et al.*, 2000, 2003). Bidgood and Papich (2005) found a similar extent of binding for MAR (22%), while a lower value (9.1%) has been reported in the product monograph (NN, 2003).

Our study demonstrated that, at standard dosages, PRA was superior to MAR against all strains in terms of early bactericidal activity as well as in terms of the overall antibacterial effect. The greater bactericidal activity of PRA was associated with higher ratios of $fAUC_{0\text{-}24\ h}$/MIC and fC_{max}/MIC. Modelling of experimental doses was performed to attain comparably high PK-PD ratios ($fAUC_{0\text{-}24\ h}$/MIC > 125, fC_{max}/MIC > 10) for both study drugs against *S. pseudintermedius* 116AD022, exhibiting MICs of 0.125 (PRA) and 0.5 (MAR). In the clinical setting, such exposure may be achievable with PRA only. The extents of killing caused by PRA and MAR were nearly comparable at 12 h ($\geq 3\ \log_{10}$), but PRA reduced viable counts faster and to a greater extent within the first 8 h of exposure. The more pronounced killing exerted by PRA may be related to the substituents at positions C-7 and C-8: PRA carries a bicyclic amine, *S,S*-pyrrolidino-piperidine, at C-7 and a cyano group at C-8, while MAR possesses a methyl-piperazine moiety at position C-7 and a six-membered (pyridobenzoxazine) ring bridging positions N-1 and C-8. Moxifloxacin also possesses an *S,S*-pyrrolidino-piperidine residue at C-7. It has been shown to have enhanced activity against staphylococci compared to earlier FQs like ciprofloxacin (Blondeau, 1999).

Substitution of C-8 has been proposed to increase the affinity of FQs for both targets, DNA gyrase and topoisomerase IV (Lu *et al.*, 2001; Drlica & Malik, 2003). Hence, the CN moiety of PRA may have contributed to the good activity of PRA against staphylococci. The C-8 moiety seems to exert an even greater activity against first and second step target mutants of Gram-negative and Gram-positive bacteria with reduced susceptibility or resistance to FQs (Lu *et al.*, 1999, 2001).

We did not detect any subpopulations with reduced susceptibility to either study drug. Consequently, static activity had prevented their outgrowth over 24 h. Our results confirmed previous reports on ratios far below 125 and 10 for $AUC_{0\text{-}24\ h}$/MIC and C_{max}/MIC, respectively, may be sufficient to restrict the selection of resistance in Gram-postive bacteria (Wright *et al.*, 2000; McKellar *et al.*, 2004). The lowest ratios preventing outgrowth of mutants with reduced FQ susceptibility were 35 and 2.7 for $AUC_{0\text{-}24\ h}$/MIC and C_{max}/MIC, respectively (corresponding to 26 and 2.1 for $fAUC_{0\text{-}24\ h}$/MIC and fC_{max}/MIC, respectively), as calculated for MAR against *S. pseudintermedius* 116AD022.

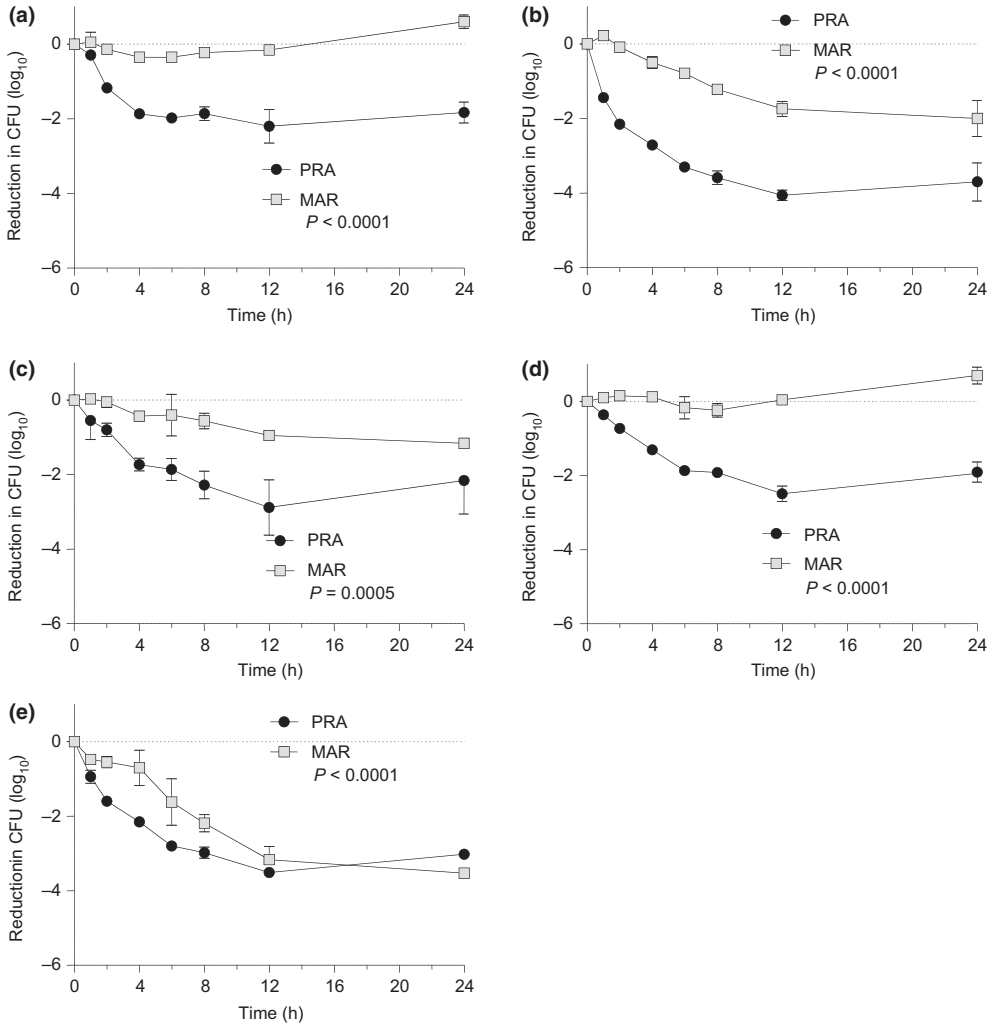

Fig. 3. Time-kill kinetics of *Staphylococcus aureus* ATCC 6538 (panel a), *S. pseudintermedius* 41JD002 (panel b), *S. pseudintermedius* 65MD004 (panel c) and *S. pseudintermedius* 116AD022 (panel d) associated with standard doses of PRA and MAR, and time-kill kinetics of *S. pseudintermedius* 116AD022 associated with experimentally high doses of PRA and MAR (panel e). Graphs are means of at least two experiments, and bars represent standard deviations.

Our findings on the relationship between MPC and the emergence of resistant mutants are in line with those reported by Homma *et al.* (2007), who investigated the emergence of mutants of *Streptococcus pneumoniae* resistant to moxifloxacin and levofloxacin, drugs used in human medicine. They found three ranges defining the emergence of resistant mutants. When the ratios of $f\text{AUC}_{0\text{-}24\ h}$/MPC and fC_{max}/MPC were >13.41 and >1.20, there was no decrease in susceptibility. Susceptibility declined, however, when $f\text{AUC}_{0\text{-}24\ h}$/MPC and fC_{max}/MPC were ≤ 0.84 and ≤ 0.08, respectively. In between, when $f\text{AUC}_{0\text{-}24\ h}$/MPC values were 2.47–6.70, and fC_{max}/MPC values were 0.20–0.60, a decrease in susceptibility was observed in one of three strains. In all of our experiments performed with PRA, ratios of $f\text{AUC}_{0\text{-}24\ h}$/MPC and fC_{max}/MPC were higher than the threshold values of >13.41 and >1.20 determined by Homma *et al.* (2007). This was also true for the experimental dose of MAR against *S. pseudintermedius* 116AD022. In contrast, all simulations associated with the standard dose of MAR resulted in $f\text{AUC}_{0\text{-}24\ h}$/MPC and fC_{max}/MPC ratios lower than 13.41 and 1.20, but still were above 0.84 and 0.08, respectively. This may provide an explanation for the absence of resistant mutants in our experiments.

Examining the activity of levofloxacin against three *S. aureus* strains, Liang *et al.* (2011) found that $\text{AUC}_{0\text{-}24\ h}$/MPC and C_{max}/MPC values of >25 and >2.2, respectively, predicted unchanged MICs. In our experiments, values obtained with PRA were above these thresholds, while those obtained with the standard dosing of MAR fell below these cut-offs. However, as subpopulations with reduced susceptibility to MAR were not detected, values of $\text{AUC}_{0\text{-}24\ h}$/MPC and C_{max}/MPC lower than those proposed for levofloxacin by Liang *et al.* (2011) may suppress re-growth of some strains and are likely to be drug-specific.

Re-growth observed in some of our experiments was most likely due to biofilm formation on the magnetic stirring bar or the wall of flask C. Parts of such biofilms may be released into the medium

during the late elimination phase which may have allowed for increased counts of bacterial cells exhibiting unchanged MICs.

PRA was developed for veterinary use, in particular for the treatment for infections in cats and dogs. One field of primary interest is the treatment for canine pyoderma caused by *S. intermedius* group isolates (including *S. pseudintermedius*). Of 101 coagulase-positive staphylococcal strains collected from cats and dogs with infections of the skin, ear or mouth between January 2004 and March 2006 by laboratories across Germany (BfT-GermVet programme), 53% were β-lactamase producing strains, while only one strain of the *S. intermedius* group was oxacillin-resistant and harboured the *mecA* gene. Resistance to tetracycline, erythromycin, clindamycin, chloramphenicole and trimethoprim/sulfamethoxazole was detected in 37, 27, 25, 22 and 13%, respectively, while resistance to enrofloxacin was observed in only 2% at that time (Schwarz *et al.*, 2007). Unfortunately, there is no published data from newer studies on the prevalence of FQ resistance among German *S. pseudintermedius* isolates. However, one can speculate that FQ resistance has increased in recent years, as a raising number of *mecA* encoding methicillin-resistant *S. pseudintermedius* (MRSP) has been identified from various geographical regions (Fitzgerald, 2009), the majority of which may be nonsusceptible or resistant to FQ (Morris *et al.*, 2006; Descloux *et al.*, 2008).

MAR at a dosage of 2 mg/kg once daily has been shown to be clinically effective in the treatment for canine pyoderma (Horspool *et al.*, 2004). Treatment with PRA also resulted in good clinical response in dogs with superficial or deep pyoderma (Mueller & Stephan, 2007; Restrepo *et al.*, 2010). In a multicentre, blinded, randomized, parallel group trial, PRA at 3 mg/kg once daily was compared with amoxicillin/clavulanic acid (AMX/CLA) at 10/2.5 mg/kg twice daily in the treatment for canine deep pyoderma. A total of 107 dogs were included into the study. PRA achieved a higher clinical remission rate (86%) than AMX/CLA (73%) and was significantly more effective in the prevention of recurrences within 2 weeks of cessation (Mueller & Stephan, 2007).

In conclusion, this study demonstrated that PRA at a dose of 3 mg/kg once daily may be expected to kill large populations of wild-type staphylococci and to prevent the emergence of FQ resistant sub-clones. Thus, the results confirm the good efficacy and low relapse rates achieved with PRA in the clinical trials for treatment for canine pyoderma (Mueller & Stephan, 2007; Restrepo *et al.*, 2010). However, prudent use of FQ is mandatory in order to restrict the selection of resistance.

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CONFLICT OF INTERESTS

MK and SB-T are partners and managing directors of Antiinfectives Intelligence GmbH, a research organization providing services to pharmaceutical companies. BK-I is an employee of Antiinfectives Intelligence GmbH. H-GW is an employee of Bayer Animal Health GmbH. DH is a scientist at Heinrich-Heine-University Duesseldorf, with no conflicts to declare.

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