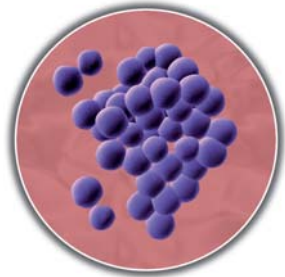


Recent advances in antimicrobial therapy of skin disease in companion animals



Dr. Peter Silley graduated in Bacteriology from the University of Birmingham in 1977. After a period in the pharmaceutical industry with Cyanamid (GB) Limited, he went to the University of Newcastle upon Tyne where he obtained his Doctorate in 1982, before moving to Northern Ireland where he held a joint appointment with the Department of Agriculture and a lectureship at the Queens University of Belfast. He continued his research interests in gastro-intestinal microbiology and subsequently joined the Glaxo group of companies, working firstly in veterinary medicine as Head of Microbiology for Glaxo Animal Health and subsequently as Senior Research Leader with Glaxo Group Research working on the development of novel anti-infective compounds in human medicine.

From April 1990 to November 2005, Peter was Research Director at Don Whitley Scientific where he has developed a highly successful contract research business largely serving the pharmaceutical industry in both Europe and the USA. In January 1999, MB Consult Limited was formed to handle the increasing demand for microbiological consultancy work. Peter split his time between the consultancy and contract research work, but since late 2005 his time is spent exclusively with MB Consult.

Dr. Silley is a member of a number of learned societies and is recent past-President of the Society for Applied Microbiology. He is Professor of Applied Microbiology at the University of Bradford and recently acted as the IFAH representative on the European VICH delegation with respect to the microbiological safety task force. In January 2005, he became an advisor to the CLSI (NCCLS) Subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST) and in January 2006 was appointed as a full voting member of VAST.

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Introduction

This presentation will focus on the currently available fluoroquinolones used in companion animal therapy, as the newest class of antimicrobials available for the treatment of skin disease. By way of introduction, I can do little better than quote from Bob Walker's chapter in the classic text book, "Antimicrobial Therapy in Veterinary Medicine" (Prescott, Baggot and Walker, 2000). Discussing the use of fluoroquinolones in dogs and cats Walker said, "Fluoroquinolones have provided small-animal clinicians with a truly exciting new class of anti-infectives. Never before have they had an antimicrobial agent with such a broad spectrum of activity as the fluoroquinolones, combined with the pharmacokinetic properties that allow for oral administration on a once-a day-basis. This has allowed clinicians to treat a larger number of patients as outpatients with more assurance that there would be owner compliance because of the once a day dosing requirement. Because fluoroquinolones can penetrate nearly every tissue in the body, these drugs can be used to treat urinary tract infections, including prostatitis caused by susceptible bacteria, respiratory infections such as rhinitis and pneumonia, includ-

ing those caused by *Bordetella bronchiseptica*; deep and superficial pyoderma, otitis media and externa, and wound infections caused by susceptible organisms; peritonitis (used in combination with metronidazole if anaerobic bacteria are suspected); osteomyelitis caused by susceptible Gram-negative aerobes; and infections caused by Mycoplasmas, such as conjunctivitis and soft tissue infections. At therapeutic doses, the fluoroquinolones have proven to be relatively safe, with few reported side effects".

The use of fluoroquinolones in companion animal medicine certainly appears to have lived up to these claims. In Denmark, the VetStat program monitors all veterinary use of medicines for animals. VetStat is based on data generated by pharmacies and from veterinarians and provides a wealth of information such as animal species for which prescriptions are dispensed, dosage and reason for the prescription. As antimicrobial drugs in Denmark are only available by prescription, this data is reliable. According to data generated by VetStat in 2003, consumption of fluoroquinolones in companion animals was substantial and this increased in 2005. Clearly use of fluoroquinolones matches the claims advocated by Walker (2000).

How do the fluoroquinolones work?

The bacterial chromosome is composed of a single, double-stranded circle of DNA which can be greater than 1000µm long. In order for it to be contained within a bacterial cell that will often be no more than 1 – 5 µm long, the chromosomal DNA undergoes tertiary folding and compaction to reduce its volume. This is achieved by: macromolecular crowding created by the high concentration of solutes in the cytoplasm; long-range DNA folding into 50-80 topologically independent domains; negative DNA supercoiling; and the presence of small DNA bending proteins (Hawkey, 2003). DNA is synthesized at a high rate, eg. 50,000 base pairs per minute per replication fork in *E. coli*, and so in order that replication occurs efficiently, the DNA strands must readily separate, and the tangles, precatenates (crossovers) and catenates (intermolecular links) must be rapidly removed. DNA topoisomerases control the topology of the chromosomal DNA to facilitate replication, recombination and expression. Two of these enzymes, DNA gyrase (topoisomerase II) and DNA topoisomerase IV, are targets of the fluoroquinolones. These two enzymes act by passing one region of duplex DNA through another and during that process, the quinolone drug traps a reaction intermediate containing quinolone, enzyme and broken DNA – the resulting complex blocks DNA replication and causes bacterial cell death (Drlica & Malik, 2003). The two targets, DNA gyrase and topoisomerase IV, share many mechanistic pro-

erties and so conclusions about drug action with one target can often be extrapolated from work with the other. DNA gyrase consists of two proteins, GyrA and GyrB, and is unique in catalyzing the negative supercoiling of DNA. It is a tetramer composed of two A and two B subunits of each monomer, encoded by *gyrA* and *gyrB* genes (Reece & Maxwell, 1991). The mechanism of DNA supercoiling by gyrase involves a transient double-strand break with a passage of another segment of DNA through the break and resealing of the broken strands. This event requires energy driven by ATP hydrolysis. The A subunits of gyrase participate in the breakage and rejoining of the DNA strands while the B subunits are responsible for the hydrolysis of ATP. Quinolones form a ternary complex with gyrase and DNA (Drlica & Hooper, 2003). The mechanisms responsible for the high affinity of drug to DNA are yet to be fully elucidated. The 4-keto and 3-carboxyl groups form hydrogen bonds with the bases of the separated DNA strand and magnesium ions play a key role for the formation of the quinolone-gyrase-DNA cleavable complex, modulating the quinolone-DNA binding process (Palumbo *et al.*, 1993). The resulting ternary complex blocks DNA replication and, for some, bacterial death occurs within hours (Drlica & Malik, 2003). Figure 1 illustrates the basic function of gyrase, and the binding of enrofloxacin with gyrase and DNA that blocks enzyme function. Complexes formed with topoisomerase IV, are similar to those with gyrase although DNA does not wrap around topoisomerase IV as it does with gyrase. As with gyrase, a topoisomerase IV alteration can block DNA cleavage while still allowing quinolone binding to complexes and quinolones reduce the ATPase

activity of topoisomerase IV (Drlca & Malik, 2003). In the absence of cleavage, quinolones enhance binding of topoisomerase IV to DNA and distortion of the DNA in the complex. As with gyrase, a major factor in complex formation is likely to be

centration of drug needed to inhibit gyrase activity *in vitro* is much higher than that necessary to exert bactericidal effects *in vivo* – this observation has been termed the “MIC paradox” (Fuchs *et al.*, 1996).

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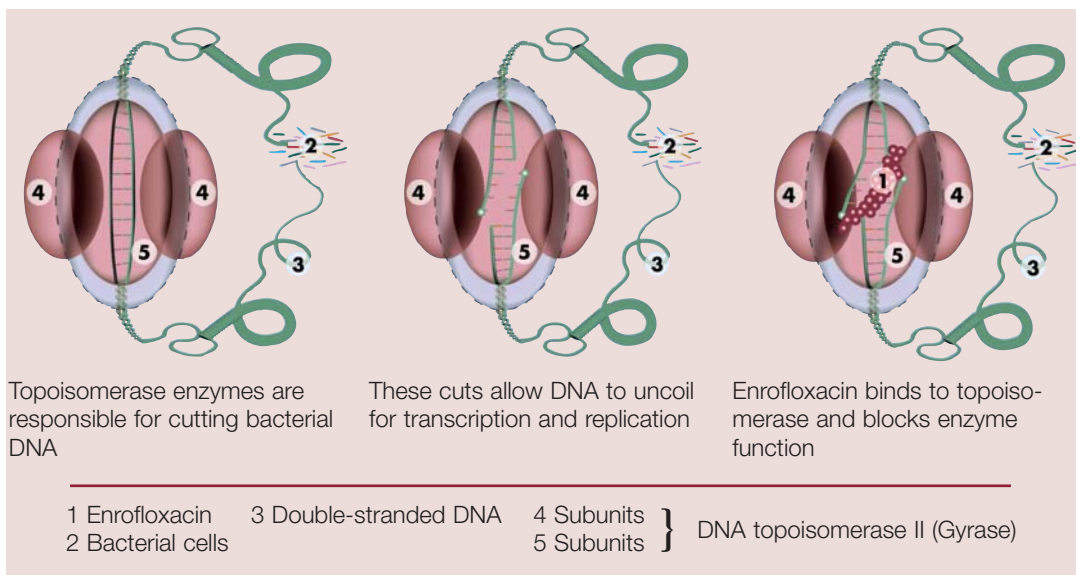
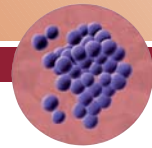


Figure 1: Fluoroquinolone mode of action

inhibition of relegation. It is now clear that the enhanced activity of third generation fluoroquinolones against Gram-positive bacteria is due to their preferential affinity for topoisomerase IV as the primary target.

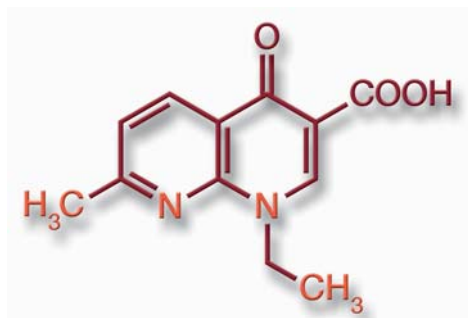
DNA replication has been demonstrated to be rapidly arrested by quinolones *in vivo* (Goss *et al.*, 1965). Quinolones also cause inhibition of other gyrase functions including relaxation, catenation, decatenation and unknotting of double-stranded DNA circles and promote double stranded DNA breakage (Fuchs *et al.*, 1996). The con-

Whilst the way fluoroquinolones work is common across the antimicrobial class, it is important to note that the new generation compounds have properties that do differentiate them from their predecessors. Körber *et al.* (2002) reported that pradofloxacin, a compound currently in development, unlike other tested fluoroquinolones, was active in the absence of protein synthesis and against non-dividing cells. This clearly suggests that pradofloxacin has additional bactericidal mechanisms compared to other fluoroquinolones. Pradofloxacin also exhibits a number of other interesting characteristics.

Spectrum of Activity

Quinolones are among the most potent antibacterial agents developed. They were first described by Leshner *et al.* (1962). The early compounds had limited *in vitro* activity against Gram-negative species. Modifications to nalidixic acid in the 1970s gave rise to similar compounds which were generally used for the oral treatment of urinary tract infections. Improvements in activity against Gram-negative and – positive pathogens followed the introduction of piperazine substitu-

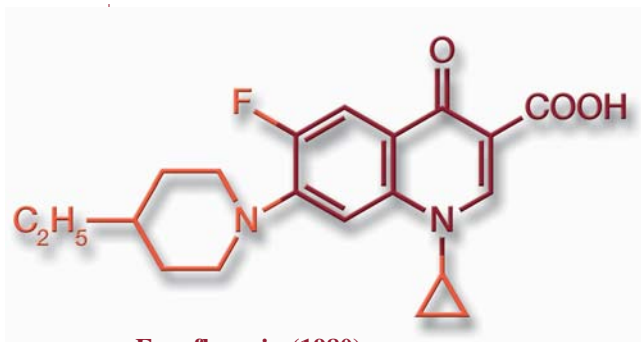
quinolones (Figure 2). Substitution of a carbon atom for nitrogen resulted in ciprofloxacin, a 1-cyclopropanyl, and ofloxacin and levofloxacin, both 1,8-cyclo compounds; all three of these latter mentioned compounds have been classified as second-generation fluoroquinolones (Blondeau, 1999). Sparfloxacin and clinafloxacin, with improved activities and pharmacokinetics, followed as extended spectrum second generation compounds. The next development was moxifloxacin resulting from a 7-azabicyclo modification that enhanced antibacterial activity and



Nalidixic Acid (1962)



Norfloxacin (1980)



Enrofloxacin (1980)

Figure 2: Development of quinolones. The discovery of the first quinolone agent, nalidixic acid, was followed by synthesis of norfloxacin and other fluorinated quinolones. Further enhancements to chemical structure lead to the second-generation fluoroquinolones, such as enrofloxacin and ciprofloxacin.

tions at position 7 of the naphthyridine core and fluorination at position 6 of the molecule, this group being commonly referred to as the fluoroquinolones. Norfloxacin was the first of the fluoro-

pharmacokinetic properties and was consequently referred to as a third generation fluoroquinolone.

The spectrum of activity of the older quinolones was essentially against *Enterobacteriaceae*,

whereas the newer fluoroquinolones have a wider spectrum including activity against many Gram-negative species (bacilli and cocci), some Gram-positive species, intracellular organisms (*Rickettsia* spp. and *Mycobacterium* spp.) and *Mycoplasma* spp. (Cambau *et al.*, 1993; Gautier-Bouchardon *et al.*, 2002; Hannan *et al.*, 1997; Rolain *et al.*, 2002; Walker, 2000). Third generation quinolones such as moxifloxacin have enhanced activity against Gram-positive bacteria relative to first and second generation compounds and good activity against anaerobes (Hawkey, 2003). Pradofloxacin

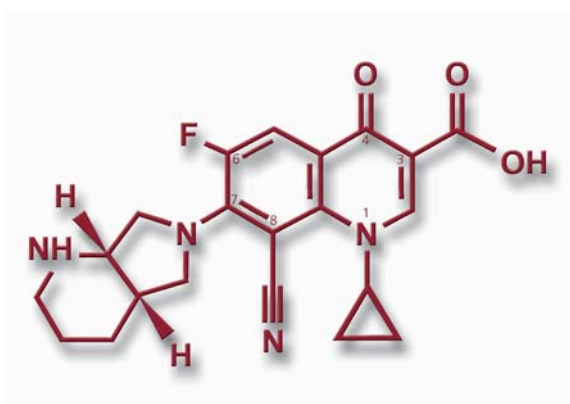


Figure 3: Chemical structure of pradofloxacin, a third-generation fluoroquinolone.

(Figure 3), developed exclusively for use in veterinary medicine, is a third generation quinolone, and therefore can be expected to show more activity against Gram-positive organisms and anaerobes than the second generation compounds such as enrofloxacin and marbofloxacin. Indeed this differentiates pradofloxacin from the compounds referred to by Walker (2000), who commented that they need to be used in combination with metronidazole if anaerobes were suspected. Pradofloxacin has demonstrated excellent anti-anaerobe activity.

Does *in vitro* data relate to the *in vivo* situation?

It is known that the antimicrobial activity of many compounds is impaired by unfavourable conditions that prevail at infection sites (Belmatoug & Fantin, 1997). This is especially relevant for dermal infections, where an environment of pus and cellular debris, granulomatous inflammation, and sequestered foci of infection may impede drug penetration and activity (Figures 4 and 5). Rubinstein *et al.* (2000), however, reported that the antimicrobial activity of moxifloxacin, a drug licensed for use in human medicine and structurally similar to pradofloxacin, was not affected by conditions likely to be found at sites of infection. The hypothesis underlying the reported study was that the presence of pus, contain-

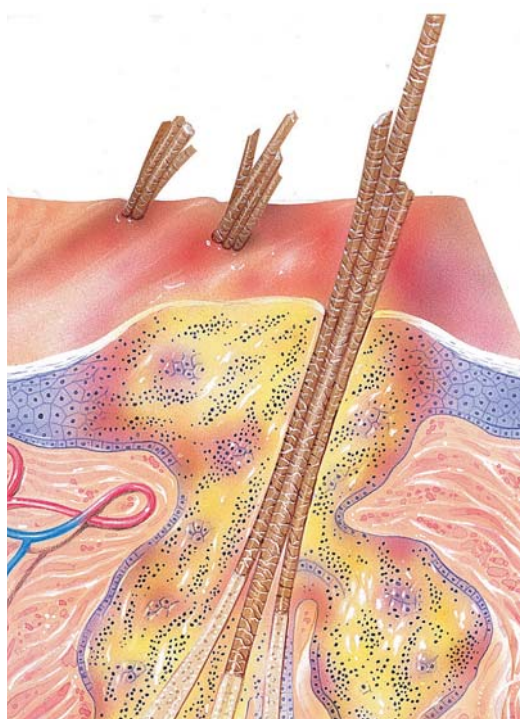
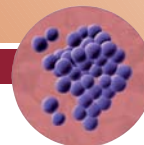


Figure 4: Deep folliculitis with purulent debris

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ing albumin, globulin and DNA, and of other bacterial debris, might inactivate the tested antimicrobials. There was no evidence whatsoever that the activity of moxifloxacin was inhibited by the conditions likely to prevail at the site of infection. These findings are consistent with data for pradofloxacin showing that pH had minimal effect on the antimicrobial activity against bacteria isolated from dogs and cats. The conditions were selected to represent as far as possible the pH range likely to be

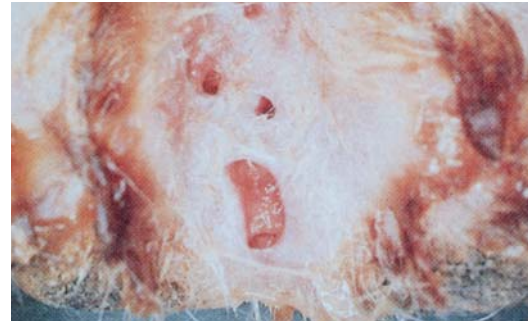


Figure 5: Severe deep pyoderma and interdigital furunculosis in a bull-terrier dog.

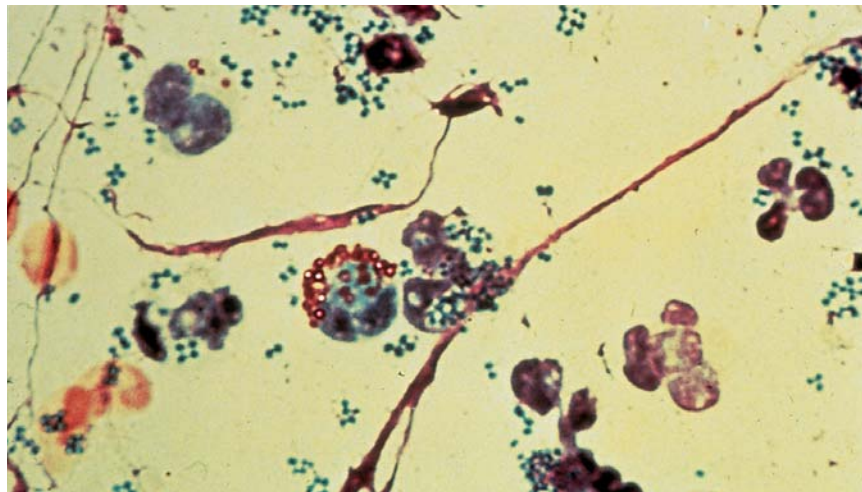


Figure 6: Cytology from dog with pyoderma, containing numerous Staphylococci and neutrophils with phagocytized bacteria

found at the site of infection whilst ensuring adequate growth of each of the tested bacterial strains. Test organisms included *E. coli*, *Pasteurella multocida*, *Pseudomonas* spp. *Streptococcus canis* and *Staphylococcus intermedius*. *Staphylococcus intermedius* is the primary pathogen involved in canine dermal infectious (Figure 6). Whilst there were marginal changes in MIC values for some organisms at some of the tested pH values, these were, with the exception of one *E. coli* strain (+2 doubling dilutions), within the acceptable margin of error for MIC determinations (i.e. ± 1 doubling dilution either side of a central value). The results of this study clearly suggested that pradofloxacin activity was not affected by extremes of pH.

Resistance in fluoroquinolones

A central feature of fluoroquinolone resistance is that it develops in a step-wise fashion with, changes considered to arise spontaneously within large bacterial populations after which the mutants will be selectively enriched by sub-optimal quinolone treatment. Fluoroquinolone resistance is

thus characterised by gradual accumulation of mutations that result in lower intracellular drug concentration and/or sensitivity of the target DNA topoisomerases; these mutations being almost without exception chromosomal. The source of such resistance will normally be a result of errors in DNA replication and repair, and mutants will normally be present in bacterial populations containing $>10^7$ cells (Drlica & Malik, 2003). It becomes clear that fluoroquinolones are not primarily the cause of the mutation, but rather they merely select for the mutation within a population thereby facilitating resistance development.

Can we prevent resistance?

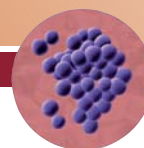
Whilst there is not the opportunity to fully characterise the fluoroquinolone resistance mechanisms, it has been well described (Drlica & Malik, 2003) that prevention of resistance development is centred on the ability to prevent clonal expansion of the mutant population. Drlica & Malik (2003) made the point that a successful strategy to restrict mutant selection is to ensure that drug concentrations are high enough to prevent the growth of selected mutants. Baquero & Negri (1997) forwarded the idea that there was a dangerous concentration range in which mutants were most frequently selected; this is considered to be the window between the MIC and the Mutant Prevention Concentration (MPC). The MPC being defined as the drug concentration at which no mutant is recovered when more than 10^{10} cells are applied to an agar plate (Dong *et al.*,

1999). Zhao & Drlica (2001) argued for a general strategy restricting the selection of antibiotic resistant fluoroquinolone mutants and presented the case for the “mutant selection window”. Drlica (2003) emphasised that the mutant selection window is an antimicrobial concentration range extending from the minimal concentration required to block the growth of wild-type bacteria (MIC) up to that required to inhibit the growth of the least susceptible, single-step mutant. The upper boundary is also referred to as the MPC. Thus, the window is the drug concentration range between the MIC and the MPC. Placing antimicrobial concentrations inside the window is expected to enrich resistant mutant subpopulations selectively, whereas placing concentrations above the window is expected to restrict selective enrichment. Since window dimensions are characteristic of each pathogen-antimicrobial combination, they can be linked with antimicrobial pharmacokinetics to rank compounds and dosing regimens in terms of their propensity to enrich mutant fractions of bacterial populations. This is an extremely important concept and needs to be considered carefully.

It is worth highlighting the principal points concerning the mutant selection window as they are relevant to the consideration of the available fluoroquinolones. In his context, pradofloxacin demonstrates particularly important properties, suggesting that its use will not easily lead to clonal expansion of resistant mutants relative to other fluoroquinolones (Wetzstein, 2005).

It is clear that if drug concentrations are below the MIC, no mutant will be enriched because selective pressure is absent, and if drug concentrations are above the MPC, then the likelihood that mutants will be selected is very low

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because a double mutation will be required for growth. It also becomes clear that if drug concentrations exceed the MPC of infecting organisms, then the expansion of resistant mutants will be restricted.

Zhao & Drlica (2001) suggested that the mutant selection window can be visualised in an idealised pharmacokinetic plot. The schematic representation in Figure 7 is taken from their

time at which the drug concentration lies inside the window. This can be accomplished if the administered drug rapidly exceeds the MPC, remains above the MPC throughout the treatment period and then rapidly is eliminated once it falls below the MPC. The rapid elimination is less important especially for a drug like pradofloxacin, which rapidly kills and thus the number of wild types and resistant mutants are likely to have been greatly reduced during the time the drug is above the MPC. The second mechanism is one in

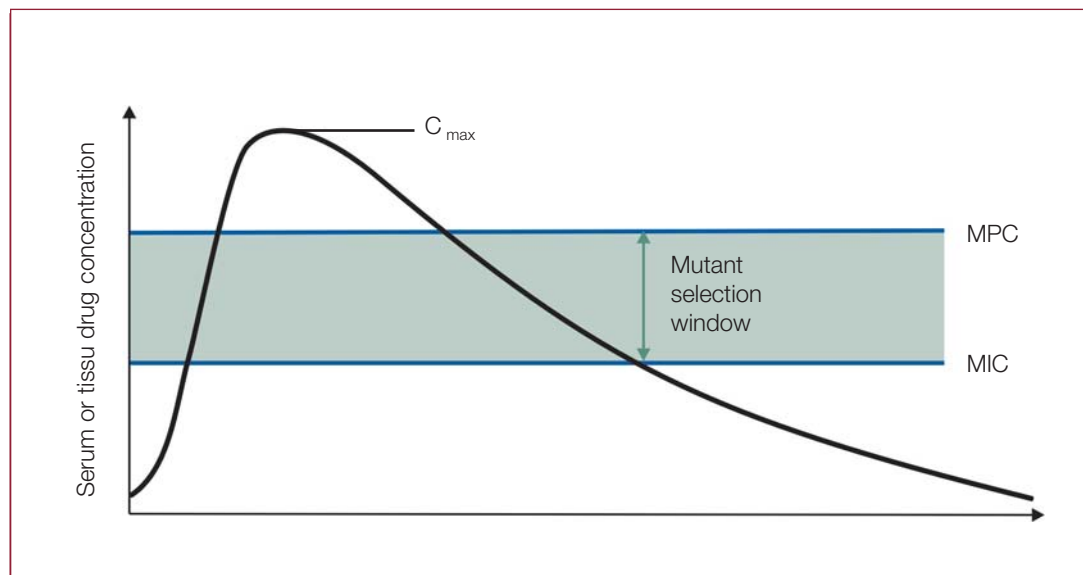


Figure 7: Idealised representation of serum or tissue drug concentration after administration of a single antimicrobial dose to a patient. MIC and MPC of the infecting organism are determined from laboratory studies. After Zhao & Drlica (2001)

review and demonstrates that if the window can be closed, mutants will not be selected.

It is clear from Figure 7 and is well argued by Zhao & Drlica (2001) that there are two means of closing the window. The first is to minimise the

which the difference between MIC and MPC is greatly reduced; this is especially the case for compounds like pradofloxacin which has very low MPC values - indeed, the best of all tested fluoroquinolones (Wetzstein, 2005). It thus becomes clear that with respect to minimising resistance development, the most effective compound may

not always be the one with the lowest MIC. Whilst Drlica (2003) emphasised that the hypothesis still awaits validation, there is good experimental support for the hypothesis (Firsov *et al.*, 2003). It is fundamentally important to provide MPC data thereby allowing an assessment of the propensity of a new active compound to give rise to resistance development. The relationship between the MIC and the MPC is fundamentally important with respect to minimising resistance development. The optimal situation is for a drug to have a very low MIC and an equally low MPC. It could therefore be argued that the best index to evaluate a drug with high intrinsic activity (low MIC) and low potential to enhance resistance (low MPC) would be to consider the value of the MIC x MPC. The elegant studies of Wetzstein (2005) revealed that the MPC of pradofloxacin against first and second step mutants was between 7- and 8-fold lower than that for enrofloxacin and marbofloxacin. In this context, pradofloxacin should possess an exceptional potential in eliminating not only large populations of wild-type *S. aureus*, *S. intermedius*, but also selected first-step resistant clones. This has been referred to as „dual targeting“ (Wetzstein, 2005). It has been stated that the ideal

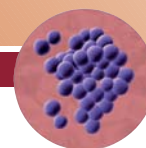
FQ should have similar affinities for both targets, DNA gyrase and topoisomerase IV (Blondeau *et al.*, 2004; Drlica & Malik, 2003), but as both enzymes differ in mode of expression as well as the molecular mechanisms, this may not be generally feasible. Nonetheless, in animals, a compound such as pradofloxacin is expected to cover both targets in *E. coli* and *S. intermedius* due to low MPC drug concentrations (Wetzstein, 2005).

Fluoroquinolones in Veterinary Medicine

Commensurate with their use, there are a number of fluoroquinolones available with indications for companion animals and, in particular, for the treatment of skin and soft tissue infections. Fluoroquinolones available in the United Kingdom are summarised below, although this list should not be considered as exhaustive for all countries.

Product	Active Ingredient	Formulation(s)	Companion Animal Claims
Baytril®	Enrofloxacin	Flavored tablet for dogs and cats 2.5% Injectable for dogs and cats 5% Injectable for dogs	Skin & wound infections Respiratory, urinary tract, gastrointestinal tract infections
Marbocyl®	Marbofloxacin	Flavored tablet for dogs and cats 1% injectable for dogs and cats	Skin & soft tissue infections, respiratory and urinary tract infections
Orbax®	Orbifloxacin	Coated tablets for dogs and cats	Wound infections and abscesses, cystitis
Ibafin®	Ibafloxacin	Tablets for dogs 3% gel for dogs & cats	Skin & soft tissue infections, upper respiratory tract infections

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