Tuning of antibacterial activity of a cyclopropyl fluoroquinolone by variation of the substituent at position C-8

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Received 3 June 2011; returned 28 June 2011; revised 11 August 2011; accepted 12 August 2011

Objectives: If substituted at position C-8 by a methoxy group, fluoroquinolones possess antibacterial efficacy considerably improved over that of C-H analogues. The new veterinary fluoroquinolone pradofloxacin bears a cyano group at C-8 and it was attempted to define the ranges of activity unfolding upon variation of this moiety.

Methods: Pradofloxacin and six analogues were subjected to MIC and mutant prevention concentration (MPC) analysis; we determined comparative activities against one wild-type and two isogenic first-step fluoroquinolone-resistant variants each of Escherichia coli and Staphylococcus aureus. Ciprofloxacin, enrofloxacin and its 8-CN analogue, the R,R-pyrrolidinopiperidine enantiomer of pradofloxacin as well as the 8-OH congener of pradofloxacin served as references.

Results: MICs were of limited utility in resolving differences in antibacterial activity. Regarding MPCs, E. coli was inhibited most effectively by ciprofloxacin. However, pradofloxacin and analogues bearing Cl or F closely matched that activity. MPCs of O-alkyl and the R,R-pyrrolidinopiperidine-substituted compounds indicated lower activities, while the 8-OH metabolite, essentially, had lost activity. Replacement of 8-H by CN, resulting in up to 7-fold reduced MPCs, was a prerequisite for high activity against the wild-type strains and first-step fluoroquinolone-resistant variants. Narrowed mutant selection windows, observed for both variants of E. coli and wild-type S. aureus, indicated an improved potential of pradofloxacin for restricting the selection of clones with reduced susceptibility.

Conclusions: Substitution of hydrogen at position C-8 of an analogue of pradofloxacin by CN provided for MPCs lower than those of 8-O-CH$_3$ and almost similar to C-8-halogenated compounds, while alkoxy substituents caused reduced activity and hydroxylation resulted in inactivation. Efficacy was co-dependent on the amine moiety located at C-7.

Keywords: pradofloxacin, enrofloxacin, ciprofloxacin, mutant prevention concentrations, structure–activity relationships

Introduction

Pradofloxacin is a new fluoroquinolone antibiotic developed for treating bacterial infections in cats and dogs. It is distinguished from enrofloxacin by a cyano group, which is attached to position C-8, as well as a bi-cyclic amine, S,S-pyrrolidinopiperidine (also contained in moxifloxacin), replacing the ethylpiperazine moiety at C-atom 7 (Figure 1). Fluoroquinolones substituted at C-8 by a methoxy group possess considerably improved bactericidal efficacy against Escherichia coli isolates with reduced fluoroquinolone susceptibility and have a lower mutant prevention concentration (MPC) for Staphylococcus aureus. $^{5,6}$

The MPC concept has been proposed in an attempt to minimize the selection of resistant clones during antibacterial chemotherapy by appropriate drug dosing. $^{5,6}$ MPC testing in vitro involves bacterial populations of $10^9$–$10^{10}$ cfu, which are generally spread onto agar plates containing a range of drug concentrations. From the numbers of regrown colonies, a population analysis profile is generated, revealing the composition of a large population with respect to fully susceptible wild-type cells and variants with reduced drug susceptibility. $^{9}$ At the MPC, no visible growth is detected, i.e. bacteriostatic activity is exerted on even the least-sensitive variant present. Thus, clonal expansion is assumed to be severely restricted. $^{1}$ While one additional mutation may confer fluoroquinolone resistance to such variants, two concurrent mutations are required for wild-type cells to continue growth, which is unlikely to occur in populations of the size used for testing. $^{7}$
Large bacterial populations are also encountered at sites of infection.\(^8,^9\) Hence, MPC conditions may have to be attained also in patients and maintained for a sufficient part of the dosing interval to produce a therapeutic effect. After peaking, drug concentrations will decline to sub-MPCs, entering the so-called mutant selection window (MSW), the lower limit of which is approximated by the MIC. At drug concentrations placed within the MSW, less susceptible variants present at the site of infection may reinitiate growth, resulting in clonal expansion.\(^10,^11\) Several animal infection models have provided strong clinical evidence in support of the MPC concept.\(^9,12–15\)

Chemical structure determines the target affinity of a drug.\(^16,^17\) Fluoroquinolones bind to protein/DNA complexes involving DNA gyrase (gyrase) and topoisomerase IV (topo IV), thus blocking replication. DNA double-strand breaks are introduced, but both strands remain covalently bound to the enzymes. At low drug concentrations, the cleavage may be reversed. In contrast, high drug concentrations cause release of the double-strand breaks and, consequently, cell death.\(^18\) For ciprofloxacin-resistant strains of \(E.\) coli, with topo IV as the secondary target; this order is reversed in \(S.\) aureus.\(^19\) However, target selectivity may vary by genus and for a particular fluoroquinolone; it has not yet been firmly established for pradofloxacin and its structural variants.

In order to elucidate the relevance of the CN group of pradofloxacin for potency, structural analogues bearing at position C-8 either hydrogen, a halogen atom (F, Cl) or an alkoxy group, such as \(O\)-CH\(_3\), \(O\)-CF\(_2\)H and \(O\)-C\(_2\)H\(_5\), were compared for antibacterial activity defined in terms of MICs and MPCs. One wild-type reference strain of \(E.\) coli and \(S.\) aureus as well as two isogenic first-step fluoroquinolone-resistant variants for each species were examined. Ciprofloxacin, enrofloxacin, the 8-CN analogue of enrofloxacin, and the 8-OH metabolite and the \(R,R\)-pyrrolidinopiperidine enantiomer of pradofloxacin served as references, the latter facilitating a brief assessment of the contribution of the C-7 amine moiety to activity.

**Materials and methods**

**Bacterial strains**

\(E.\) coli ATCC 8739 and \(S.\) aureus ATCC 6538 were selected as model wild-type strains. The MICs and MPCs of pradofloxacin for these strains, also covering clinical isolates as well as \(Staphylococcus\) \((\text{puccip})\) \(\text{intermedius}\), have been reported before.\(^19\) Two isogenic first-step fluoroquinolone-resistant variants of \(E.\) coli had single point mutations in \(gyrA\), causing an exchange in subunit A of gyrase (GyrA) of either Ser-83\(\rightarrow\)Leu or of Asp-87\(\rightarrow\)Tyr. Mutant strains of \(S.\) aureus had a single point mutation in \(parC\) (\(\text{gprA}\)), either causing an exchange in topo IV subunit A (\(\text{ParC}\)) of Ser-80\(\rightarrow\)Phe or of Glu-84\(\rightarrow\)Lys. The second subunits of these heterodimeric enzymes, GyrB and ParE, respectively, were wild-type. All strains were kindly provided by P. Heisig, University of Hamburg, Germany.

**Fluoroquinolone drugs and analogues**

Reference standards of pradofloxacin, enrofloxacin and ciprofloxacin with purities of \(\geq 99.5\%\) were employed throughout. Analogues of pradofloxacin carrying at position C-8 either H \(\text{(BAY Y 3114)}\), F, Cl \(\text{(BAY Y 3118)}\), O-CH\(_3\) \(\text{(moxifloxacin)}\) or O-C\(_2\)H\(_5\), the \(R,R\)-pyrrolidinopiperidine analogue of pradofloxacin as well as the 8-CN analogue of enrofloxacin (with purities of \(\geq 98.6\%\)) were obtained from the collection of fluoroquinolone standard compounds at Bayer AG. The 8-OH and O-C\(_2\)H\(_5\) analogues of pradofloxacin \(\text{(purity} \geq 93\%)\) were kindly donated by U. Petersen (formerly of Bayer AG).\(^11\) Structures were confirmed before use by \(\text{\(^1\text{H}\)-nuclear magnetic resonance}\) spectroscopy and high-resolution mass spectrometry, as described previously.\(^20–22\) The compounds were dissolved in sterile, double-distilled water \(\text{(500 mg/L)}\). The exact drug concentrations of the reference standards were verified and monitored over time of use by standard HPLC analysis,\(^23\) while the other stocks had to be set up gravimetrically, according to certified purity. All solutions were stored at room temperature and protected from light.

**Culture conditions and MPC determination**

Overnight cultures in tryptic soy broth \(\text{(Merck, Darmstadt, Germany)}\) were harvested by centrifugation, washed and resuspended in physiological saline, as described previously.\(^19\) For MPC testing, bioassay dishes with a surface area of \(530 \text{ cm}^2\) were filled with 155 mL of Difco\(^{24}\)Balanced Sensitivity Test Medium, pH 7.4 \(\pm\) 0.2 \(\text{(Becton, Dickinson & Co., Sparks, MD, USA)}\), supplemented with an appropriate concentration of the test compound. Following 20 h of drying at 36 \(\pm\) 1°C in the dark, aliquots of 0.8 mL of a concentrated cell suspension, containing \(4 \times 10^8 \pm 2 \times 10^8\) cfu, were streaked onto each plate. After 10 min of drying, the plates were put into a polypropylene bag and incubated at 36 \(\pm\) 1°C in the dark to prevent light-induced drug decomposition. Thus, the agar volume \(\text{(2 mL)}\) and drug concentration could be kept constant over 14 days of incubation. Bacterial regrowth was monitored by colony counting, including slow-growing small colony variants (SCVs). The upper limit of detection was set at 4000 colonies per plate. To determine MPCs graphically, the total cfu \(\text{(geometric mean; SD)}\) recovered in three to five independent experiments for each compound/strain combination were plotted over the respective drug concentration. Median values were used to express the relative activities, if the MIC or MPC had been stated as concentration ranges.\(^18\) Alternative media, used to verify the MPCs of the 8-H analogue versus pradofloxacin for wild-type \(S.\) aureus, comprised Mueller–Hinton agar \(\text{(Oxoid Ltd, Basingstoke, UK)}\) and tryptic soy agar \(\text{(Merck, Darmstadt, Germany)}\).
MIC determination

MICs were determined by agar dilution employing Iso-Sensitest agar, pH 7.4 + 0.2 (Oxoid), but otherwise in accordance with the CLSI method, as described previously. The size of the inoculum was in the order of 10^4 to 5 × 10^4 cfu per spot. MICs were read after 20 (wild-type strains) and 44 h (first-step variants) of incubation at 36 ± 1°C.

Results

MICs for wild-type strains

MICs of pradofloxacin were similar to those of its 8-H analogue and only marginally higher than the MICs of C-8-halogenated compounds or of ciprofloxacin for E. coli (Table 1). Analogues carrying an alkoxy group at C-8 had elevated MICs, increasing with substituent size. Very high MICs of the 8-OH analogue indicated inactivity; hence, it was excluded from MPC analysis. Even for the R,R-pyrrolidinopiperidine enantiomer of pradofloxacin, just slightly increased MICs were determined, which, however, resembled the MICs of enrofloxacin. The MIC of enrofloxacin amounted to twice that of ciprofloxacin for E. coli, but to one-quarter of that for S. aureus. Notably increased MICs were observed for the 8-CN analogue of enrofloxacin.

Population analysis profiles

Graphs revealing the composition of large populations of both wild-type strains upon exposure to enrofloxacin are depicted in Figure 2; respective profiles for pradofloxacin have been reported previously. Populations comprise colonies of regular size and

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituent at C-8</th>
<th>E. coli ATCC 8739</th>
<th>S. aureus ATCC 6538</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (mg/L)</td>
<td>MPC (mg/L)</td>
<td>MPC/MIC</td>
</tr>
<tr>
<td>Pradofloxacin</td>
<td>-CN</td>
<td>0.015–0.03</td>
<td>0.2–0.25</td>
</tr>
<tr>
<td></td>
<td>-H</td>
<td>0.015–0.03</td>
<td>0.4–0.45</td>
</tr>
<tr>
<td></td>
<td>-O-CH₃</td>
<td>0.06–0.125</td>
<td>0.8–0.9</td>
</tr>
<tr>
<td></td>
<td>-O-CF₂H</td>
<td>0.125–0.25</td>
<td>2–2.2</td>
</tr>
<tr>
<td></td>
<td>-F</td>
<td>0.15</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>-Cl</td>
<td>0.15</td>
<td>1.8–0.18</td>
</tr>
<tr>
<td></td>
<td>-CNP</td>
<td>0.06</td>
<td>1.75–2</td>
</tr>
<tr>
<td></td>
<td>-OH</td>
<td>4–8</td>
<td>ND</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>-H</td>
<td>0.03–0.06</td>
<td>0.3–0.35</td>
</tr>
<tr>
<td></td>
<td>-CN</td>
<td>0.125–0.25</td>
<td>1.5–1.75</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>-H</td>
<td>0.015</td>
<td>0.13–0.15</td>
</tr>
</tbody>
</table>

ND, not done; Sa, Staphylococcus aureus; Ec, Escherichia coli.

Quotients of MPC/MIC, representing the MSW, were calculated by dividing the lower values of MPC by the lower MIC, and the upper values, Similarly. Notably broadened MSWs are shown in italics. Similarly, the most divergent affinities for the second targets in S. aureus versus E. coli are also indicated.

aR,R-pyrrolidinopiperidine enantiomer of pradofloxacin.
morphology as well as SCVs. The initial steep drop in cfu at concentrations around the MICs indicated inhibition of the most sensitive target. However, a subpopulation of \( \approx 10^5 \) cfu still formed colonies, apparently containing a resistant first target. Colony formation of such variants was successively blocked (likely, via inhibition of a second target) upon increasing the drug concentration. For \( E. coli \) and \( S. aureus \), MPCs of 0.3–0.35 and 3–3.5 mg/L enrofloxacin, respectively, were determined (Figure 2). Colonies of regular size mostly represented the minority in the populations of both species recovered. SCVs were particularly prominent in specific drug concentration ranges. For \( E. coli \), SCVs even determined the MPC at 1.5-fold the value that would have been derived from colonies of regular size. The heterogeneity in susceptibility encountered in the large populations amounted to \( \approx 10 \) times the MIC (Figure 2).

MPCs of pradofloxacin and its structural analogues and reference compounds

The curves depicted in Figure 3 represent the right-hand part of the population analysis profiles shown in Figure 2, relevant for MPC determination and indicating total cfu; the actual MPC values are compiled in Table 1. In most cases, the curves offer more detailed information on the size of the population placed inside the MSW as well as the range of drug concentrations bringing about the decline in cfu towards the MPC can be identified. To enhance readability, the compounds are listed in the order indicated in Table 1 (except for the 8-OH metabolite). PRA, pradofloxacin; ENR, enrofloxacin; CIP, ciprofloxacin.

Figure 3. Determination of the MPCs of pradofloxacin and its structural analogues and reference compounds for \( S. aureus \) ATCC 6538 and \( E. coli \) ATCC 8739. The graphs represent the right-hand (lower) part of the population analysis profiles as shown in Figure 2. The MPC was attained at the intercept between the graphs and the lower detection limit. In most cases, the size of the subpopulation(s) placed inside the MSW as well as the range of drug concentrations bringing about the decline in cfu towards the MPC can be identified. To enhance readability, the compounds are listed in the order indicated in Table 1 (except for the 8-OH metabolite). PRA, pradofloxacin; ENR, enrofloxacin; CIP, ciprofloxacin.

In contrast, the MSW was narrowest for pradofloxacin and its 8-Cl analogue.
and 54, respectively, revealed vast differences and a particularly low affinity for the second target in *S. aureus*. For pradofloxacin, a value of 2.4 was comparably close to unity.

### Contribution of SCVs to MPCs

Generally, MPCs were increased by >10% due to SCVs, except for enrofloxacin (Figure 2). No such increase was observed for *E. coli* with analogues carrying H or F. For *S. aureus*, due to SCVs, MPCs of the 8-Cl and R,R-pyrrolidinopiperidine analogues were increased by 25% and 40%, respectively. If read after 5 (*E. coli*) or 7 days (*S. aureus*) instead of 14 days of incubation, MPCs would have been ~20%–30% below the values listed in Table 1.

### Significance of the 8-CN group for efficacy against first-step fluoroquinolone-resistant variants

MICs and MPCs of pradofloxacin and its 8-H analogue for isogenic first-step fluoroquinolone-resistant variants are shown in Table 2, along with reference data for the wild-type strains and enrofloxacin as the reference drug (Table 1). Compared with the wild-type, MICs of pradofloxacin and its 8-H analogue were increased 4- to 8-fold, with similarly increased MPCs for *S. aureus*. Nonetheless, MPCs of pradofloxacin were the lowest throughout and about half of those of the 8-O-CH₃ analogue for *S. aureus* (data omitted from Table 2).

Compared with the wild-type, increases in the MPC for *E. coli* Ser-83→Leu were 14-fold for pradofloxacin, 22-fold for its 8-H analogue and 42-fold for enrofloxacin (and even 46-fold for ciprofloxacin, with MPCs of ciprofloxacin of 0.1–0.15 mg/L for the wild-type strain and 5.5–6 mg/L for this first-step variant; data not shown). Such increases were half as pronounced for the second variant, *E. coli* Asp-87→Tyr, amounting to 7-, 11- and 19-fold, respectively (Table 2). For both first-step variants of *E. coli*, and in contrast to the wild-type, the MPCs of the 8-H analogue were slightly lower than the MPCs of enrofloxacin, suggesting an activity slightly enhanced due to the S,S-pyrrolidinopiperidine substituent.

Quotients of MPC/MIC for first-step fluoroquinolone-resistant clones indicated the following: (i) for pradofloxacin, MSWs widened for *E. coli* but slightly narrowed for *S. aureus* (as compared with the wild-type); (ii) for the 8-H analogue, MSWs widened for *E. coli* Ser-83→Leu, but narrowed for *S. aureus* Glu-84→Lys (as compared with the wild-type); (iii) for the 8-H analogue versus pradofloxacin, there was a widened MSW for the 8-H analogue in *E. coli* Ser-83→Leu, but drastically widened MSWs for both *S. aureus* variants; (iv) for pradofloxacin versus enrofloxacin, MSWs of pradofloxacin were narrowed for *E. coli* but similar in *S. aureus*; and (v) for enrofloxacin versus the 8-H analogue of pradofloxacin, MSWs were similar for both *E. coli* clones, but considerably narrowed for enrofloxacin in *S. aureus*.

### Discussion

Previously, a low antibacterial activity has been assigned to fluoroquinolones substituted at position C-8 by a CN group. Choosing enrofloxacin as a starting point, the introduction of CN at C-8 confirmed those observations in terms of MICs and MPCs. However, an additional exchange of the ethylpiperazine for the bi-cyclic S,S-pyrrolidinopiperidine moiety provided the highly active pradofloxacin. Evidently, the amine group impacts on activity just as the substituent at position C-8 does. The latter was focused on herein, with six structural analogues of pradofloxacin analysed.

Recently, X-ray crystal structures of cleaved complexes, comprising DNA, top IV from *Streptococcus pneumoniae* or *Acinetobacter baumannii*, and levofloxacin or moxifloxacin, respectively, have been published. The latter structure has been modelled to account for gyrase of *Mycobacterium smegmatis*. In such ternary complexes, the essential 3-carboxyl and 4-oxo groups interact with helix IV of ParC/GyrA, while the C-7 substituent interacts with ParE/GyrB. Hence, some of our observations may be compared with structure–activity relationships reported for *M. smegmatis*. However, the MIC and MPC values presented herein should be interpreted tentatively as reflecting target affinities, because the compounds may differ in cell penetration, target selection, affinity for various drug efflux systems and non-specific binding to cellular constituents.

The MICs of pradofloxacin and its C-8 analogues were quite similar for wild-type reference strains of *E. coli* and *S. aureus*; thus, offering a limited diagnostic potential, in particular, for defining the significance of the 8-CN group for activity.
MICs revealed that, in order to match the activity against E. coli of the ‘gold standard’ ciprofloxacin, analogues of pradofloxacin needed to carry at C-8 either a halogen atom or the CN group (in combination with the S,S-pyrrolidinopiperidine moiety). However, C-8 halogen-substituted fluoroquinolones are known to cause severe phototoxic reactions.\textsuperscript{20} In contrast, O-alkyl-substituted analogues exhibited considerably increased MICs, as has been observed before.\textsuperscript{30} The analogue carrying a hydroxyl group at C-8 has been identified during in vitro fungal degradation of pradofloxacin.\textsuperscript{22} Its residual antibacterial activity for both species was negligible (Table 1), as has been observed before with 8-OH-enrofloxacin.\textsuperscript{21} This is of considerable mechanistic and ecological interest, and awaits explanation at the molecular level. The 8-Cl analogue provided the lowest MIC for S. aureus. Otherwise, C-8 substituents caused relatively little variation in the MICs (affinity for topo IV), in contrast to for E. coli (affinity for gyrase).

Remarkably, on the basis of the MICs, the increase in activity, caused by substituting 8-H with the CN group or the R,R-pyrrolidinopiperidine with the S,S-enantiomer, could either not be resolved at all or not resolved convincingly, respectively, for both species. Obviously, the diagnostic potential of the MIC is limited by the 2-fold dilutions used for its determination and by the inoculum size of 10⁴ cfu per spot or 5×10⁴ cfu per vial, as are generally used in agar dilution and microbroth dilution assays, respectively.\textsuperscript{23} Such cfu represent the most abundant, susceptible and homogeneous fraction encountered in large bacterial populations (see Figure 2). The increase in antibacterial activity conferred by the CN group could only be diagnosed in terms of MPCs, i.e., affinity for the secondary target.

In E. coli, increasing the size of the amine substituent of ciprofloxacin (piperazine) to ethylpiperazine (enrofloxacin) and S,S-pyrrolidinopiperidine (8-H analogue of pradofloxacin) reduced activity (increased MICs and MPCs), and only the additional 8-CN group provided for maximum activity. The piperazine ring of ciprofloxacin already had provided for optimal interaction with gyrase. The opposite effect was reported for ciprofloxacin and enrofloxacin in wild-type M. smegmatis.\textsuperscript{28} However, activity (in terms of MICs) was highest for a series of 8-O-CH₃-substituted fluoroquinolone analogues against both the wild-type and eight first-step fluoroquinolone-resistant mutants, and it was further increased if the piperazine substituent carried an alkyl group, in particular 3'-methyl; N-ethyl or N-isopropyl substituents caused reduced activity.\textsuperscript{28} 8-F-substituted analogues were generally less active than compounds substituted by 8-O-CH₃,\textsuperscript{28} which also is opposite to our findings.

In S. aureus, increasing the size of the amine moiety by adding an ethyl group to the piperazine ring provided for a reduced MIC. Replacement of the ethylpiperazine by the S,S-pyrrolidinopiperidine moiety resulted in maximum activity in terms of MICs. Another example is given by wild-type M. smegmatis: the 8-H analogue of pradofloxacin (BAY Y 3114) had the highest activity (lowest MIC), which was reduced to half upon substitution by 8-O-CH₃, but then restored by exchanging the amine for a 3'-ethylpiperazine residue.\textsuperscript{27} This exemplifies the interdependence of the substituents at C-7 and C-8.

MPC determination involves the less susceptible variants present in large populations and addresses drug affinity for the second target. Avoiding 2-fold dilutions, as depicted in Figure 3, evidently increases the diagnostic potential. In E. coli, the lowest MPC was determined for ciprofloxacin, carrying a piperazine ring at C-7 and hydrogen at C-8 (Figure 1), followed by almost similar MPCs of halogen-substituted analogues and pradofloxacin. Thus, activity was increased by substituents that reduce electron density in the aromatic core. In contrast, angular O-alkyl residues at C-8 were associated with considerably increased MPCs.

Notably, an exchange of the ethylpiperazine moiety of enrofloxacin for the S,S-pyrrolidinopiperidine residue (providing the 8-H analogue) failed to impact the MPCs for both wild-type strains, although this enantiomer, finally, was obligatory for effective inhibition of the second target in both species, in particular for E. coli (topo IV). An MPC of enrofloxacin twice that of even the R,R-pyrrolidinopiperidine analogue of pradofloxacin indicated a positive effect of the size of the amine substituent on the activity against S. aureus. However, upon substitution of 8-H for CN, the MPC was reduced 7-fold. The MPC of the 8-H analogue (Table 1) was due to a small refractory subpopulation (Figure 3). Nonetheless, the increase in activity could be verified by employing two alternative media and, therefore, should reflect increased target affinity, which needs to be verified at the level of the isolated target enzymes.

MSWs were particularly widened for analogues carrying at C-8 either hydrogen or O-C₂H₅, the R,R-pyrrolidinopiperidine (E. coli), or O-CF₂H (S. aureus). The narrowest MSWs in S. aureus were determined for pradofloxacin and its 8-CN analogue. It should be mentioned that an 8-CH₃ analogue of pradofloxacin was not available. However, an 8-CH₂β-quinolizinone (2-pyridone)\textsuperscript{30} analogue of pradofloxacin maintained very high activity (low MICs) for wild-type and first-step fluoroquinolone-resistant strains of E. coli and S. aureus, while a 5-methyl analogue of pradofloxacin exhibited up to 8-fold reduced activity (H.-G. Wetzstein, unpublished observations).

For our first-step fluoroquinolone-resistant variants, the MICs and MPCs were markedly increased over those determined for wild-type strains. This is likely due to the reduced interaction of the 3-carboxyl and 4-oxo groups with the target site, and may indicate a positioning of pradofloxacin similar to that of levofloxacin\textsuperscript{25} and moxifloxacin.\textsuperscript{26,27} Compared with enrofloxacin, the MPCs of pradofloxacin were 3- to 4-fold lower in S. aureus than in E. coli and 7- to 8-fold lower in S. aureus; indicating a notably improved potential for inhibiting even a third target at drug concentrations of ~2 mg/L, which are attainable in infected tissues.\textsuperscript{31,32} At the same time, this suggests an improved potential of pradofloxacin for inhibiting first-step fluoroquinolone-resistant variants contained in large populations of wild-type strains; for E. coli, this activity may even exceed that of ciprofloxacin. Reduced binding at the carboxyl/oxa site was likely compensated for by the affinity increase gained due to the CN and amine substituents.

Replacement of the ethylpiperazine group of enrofloxacin for S,S-pyrrolidinopiperidine was associated with only slightly narrowed MSWs in the E. coli variants, but much widened MSWs in the S. aureus variants. In S. aureus, the affinity for the second target (possibly gyrase) was drastically reduced and only restored by adding the 8-CN group. Similarly, a larger amine had caused suboptimal target interaction for first-step variants of M. smegmatis. Activity for BAY Y 3114 was doubled upon the introduction of the 8-O-CH₃ substituent and doubled again in the presence of a 3-aminomethyl-pyrrolidine residue at C-7, possibly due to an optimized positioning of the amino group towards Glu-501 of ParE/GyrB at the binding site (see Figure 6A in Malik et al.\textsuperscript{21}). For a primary amine analogue of pradofloxacin,
Tuning fluoroquinolone activity by substitution of C-8

reported by Petersen et al. (see Figure 14.13, centre of the upper row), the positioning of the amino group may be similar, as this compound also exhibited higher activity (MICs lower than those of pradofloxacin) against S. aureus (H.-G. Wetzstein, unpublished observations). The 8-CN substituent present in pradofloxacin provides for an unprecedented potential for minimizing the selection of clones of antibiotic resistance, as suggested by MPCs. Narrowed MSWs may indicate an improved likelihood of coverage of a pathogen for the dosing interval and to predict clinical response. In the most recent pharmacodynamic models, conventional MPCs may be useful in drug lead optimization, supplementing MICs, which may be combined with pharmacokinetics, ideally, the time course of free drug concentrations in serum or tissues, to estimate the likelihood of coverage of a pathogen for the dosing interval and to predict clinical response. In the most recent pharmacodynamic evaluations of MPC, in vivo pharmacokinetics are simulated by flow-through models, facilitating the direct quantification of all drug effects exerted on a large population. Such studies now need to be performed with pradofloxacin.

Pharmacokinetic studies in dogs and cats suggest that: (i) concentrations exceeding MPCs should be attained in patients infected with wild-type E. coli and S. aureus; (ii) MPCs are maintained for a sufficient part of the dosing interval (see below); and (iii) even large populations of first-step variants may become therapeutically accessible upon adjusting the dose. According to the most recent findings in a rabbit infection model, conventional in vitro MPCs are likely to overestimate the time span for which drug concentrations need to exceed the MPC: half of the dosing interval may be sufficient. A comparison of the MPCs of wild-type S. aureus over E. coli implies that pradofloxacin at the lowest concentration exhibited the most closely balanced activity against our Gram-positive and Gram-negative model pathogens. Therefore, in addition to high clinical efficacy, as suggested by MPCs, narrowed MSWs may indicate an unprecedented potential for minimizing the selection of clones of antibiotic resistance with reduced susceptibility during therapy. The 8-CN substituent present in pradofloxacin provides for a combination of optimized antibacterial activity, a moderate or absent in vivo phototoxic potential as well as the absence of retinal toxicity.

Acknowledgements

Part of this work was presented at the Forty-sixth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, USA, 2006 (Abstract C1-46). The technical assistance of Nora Schröter, Angelika Seitz, Sven Feisel and Bernd Nitzgen is gratefully acknowledged. We also thank Peter Opdam and Darren Trott for critical reading of the manuscript prior to submission.

Funding

This work was funded by Bayer Animal Health GmbH.

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