F1-1968

Low Propensity for Development of Resistance to MCB3681, the Active Moiety of Oxaquin (MCB3837), in Gram-positive Bacteria with Vancomycin-, Linezolid-, Methicillin- and/or Ciprofloxacin Resistances.

Sabine Schubert, Axel Dalhoff University-Hospital Schleswig-Holstein

San Francisco, CA, USA

September 27-30, 2006

46th ICAAC

University-Hospital Schleswig-Holstein
Campus Kiel
Institute for Infection Medicine
Brunswiker Str. 4
D - 24105 Kiel

ABSTRACT

Objective: MCB3681 is the active metabolite of the water soluble prodrug MCB3837 (Oxaquin); Oxaquin is derived from a quinolone and an oxazolidinone, linked with a spacer. MCB3681 was identified as a drug candidate for treatment of infections caused by multi-resistant and difficult to treat Gram-positive bacteria. As it is important to characterise the propensity for resistance development in the early phases of drug development, we quantified the potential of MCB3681 to select for singlestep- or multistep-resistance in selected strains with multiple resistance genotypes.

Methods: Thirteen strains of Streptococci, Staphylococci, and Enterococci with and without genotyped resistances to vancomycin, linezolid and/or ciprofloxacin were tested. First, the bacteria were exposed for 18h at 37°C to MCB3681 concentrations ranging from two- to eight-times the individual MICs. The single step resistance frequencies (ssrf) were calculated by dividing the numbers of colonies growing on MCB3681 containing agar plates by the inoculum. Second, bacteria were exposed to sub inhibitory concentrations of MCB3681 for 7 days to elicit multi step resistance (msr). MICs were recorded daily.

Results: No spontaneously resistant colonies were isolated following exposure of the fully susceptible or ciprofloxacin-, penicillin- and macrolide resistant S. pneumoniae isolates to two- to eight- times their MICs of MCB3681. The ssrf's in Enterococci were low ($1x10^{-8}$ to $1x10^{-7}$), even in the vanA- or vanB-, and linezolid-resistant strains. Likewise, the ssrf's were low in the MSSA and MRSA reference strains of S. aureus, as well as in the VISA strain upon exposure to four-times their MICs of MCB3681 These numbers are in the same range as previously tested comparators like e.g. Moxifloxacin. Higher ssrf ($3.4x10^{-4}$) was recorded in a single MRSA clinical isolate, due to unknown reasons. The msr's were low, too. The only strains showing a 2- to 3-fold increase in MICs of MCB3681 during the first three passages were vancomycin-resistant enterococci carrying the vanA or vanB glycopeptide resistance gene. In all other strains, the MICs increased by less than two dilution steps during the first three passages, confirming the low propensity for resistance development.

Conclusions: Overall, spontaneous emergence of resistance to MCB3681s rare even in strains with multiple resistance mechanisms.

INTRODUCTION

MCB3681, the active metabolite of the prodrug MCB3837, is a novel antibacterial combining the structural moieties of an oxazolidinone- and a quinolone. MCB3681 was identified as a potential new antimicrobial agent for parenteral treatment of infections caused by Gram-positive bacteria.

The introduction of antibacterial agents into clinical practice has been followed by an almost immediate development of resistance, which has increased rapidly since the previous years. It is a likely consequence of exposure to any antibacterial drug-class that resistance will develop.

Consequently, it is important to characterise the propensity for resistance development in the early phases of drug development. Therefore, the spontaneous as well as multistep selection of MCB3681-resistant subpopulations has been studied in difficult to treat gram-positive pathogens. The test organisms were selected out of different strain collections with the aim to incorporate well characterised isolates, acknowledged reference strains and strains with genetically defined resistance mechanisms.

Thus, this panel of test strains represents the epidemiologically relevant resistance patterns among the key target pathogens to be treated with MCB3681. However, this panel does not represent the clinically relevant frequency of resistances among the species studied, as the strains were selected on the basis of their resistance phenotype or genotype.

MATERIALS AND METHODS

Test strains and media

The following 13 Gram-positive strains were tested:

- *Staphylococcus aureus* ATCC 29213 (MSSA)
  Methicillin-susceptible wild type reference strain for resistance testing (vancomycin-, linezolid- and ciprofloxacin-susceptible)
- *Staphylococcus aureus* ATCC 33593 (MRSA)
  Methicillin-resistant reference strain (vancomycin-, linezolid- and ciprofloxacin-susceptible)
- *Staphylococcus aureus* NRS 119 (MRSA)
  Methicillin-resistant clinical isolate (vancomycin susceptible, linezolid-, and ciprofloxacin-resistant)
- *Staphylococcus aureus* VISA Mu 50 (VISA)
  Methicillin-resisistant clinical isolate (vancomycin-intermediate, linezolid-susceptible, ciprofloxacin-resistant)
- *Staphylococcus aureus* P8 (MRSA)
  Methicillin-resistant clinical isolate (vancomycin-, linezolid- and ciprofloxacin-susceptible)
- *Enterococcus faecalis* ATCC 29212
  Reference strain for suceptibility testing (vancomycin-, linezolid- and ciprofloxacin-susceptible)
- *Enterococcus faecalis* ATCC 51299 (VRE)
  Vancomycin-resistant reference strain for susceptibility testing, clinical isolate (vancomycin-resistant, linezolid- and ciprofloxacin-susceptible)
- *Enterococcus faecalis* V 583 (VRE, Van-B)
  Vancomycin-resistant isolate, international reference strain (vancomycin-resistant, linezolid- and ciprofloxacin-susceptible), Van-B type of glycopeptide-resistance
- *Enterococcus faecalis* UW 3696 (LRE)
  Linezolid-resistant clinical isolate (vancomycin-susceptible, linezolid- and ciprofloxacin-resistant)
- *Enterococcus faecium* BM 4147 (VRE, Van-A)
  Vancomycin-resistant isolate, international reference strain (vancomycin-resistant, linezolid-susceptible, ciprofloxacin-resistant), Van-A type of glycopeptide-resistance
- *Enterococcus faecium* UW 3695 (LRE)
  Linezolid-resistant clinical isolate (vancomycin-susceptible, linezolid- and ciprofloxacin-resistant)
- *Streptococcus pneumoniae* ATCC 49619
  Reference strain, clinical isolate (penicillin-, vancomycin-, linezolid- and ciprofloxacin-susceptible)
- *Streptococcus pneumoniae* Bay 19397 (fluoroquinolone resistant)
  Fluorochinolone resistant laboratory strain (penicillin-resistant, vancomycin- and linezolid-susceptible, ciprofloxacin-resistant), gyr A mutation.

Brain Heart Infusion (BHI) broth or agar, (Oxoid GmbH, Wesel, Germany) supplemented with 5% sheepblood, (Oxoid GmbH, Wesel, Germany) was used for growth of S pneumoniae. Bacteria were cultivated at 36°C +/-1°C in ambient air for 18h.

The inoculum was prepared from frozen stock-culture strains. The final inocula of the test-strains were adjusted with BHI broth by using a No.1 McFarland standard ($1x10^8$ CFU/ml); range from $1x10^8$ to $1x10^9$ CFU/ml.

Singlestep resistance testing

The singlestep resistance testing was done as outlined in (1). To characterise the emergence of spontaneous resistance the MCB3681-resistant variants were isolated by spreading the inoculum over BHI-agar plates incorporating the study drug at 2x, 4x, 8x MICs of the individual test organisms. 50 plates per strain and MCB3681-concentration have been used to screen for resistance, so that even the occasional emergence of resistant colonies (which are missed if too few plates are used) will be detected.

Following an overnight incubation at 36°C +/-1°C the frequency of the spontaneous resistance was determined by colony counting.

Multistep resistance testing

The susceptibilities of the test strains against MCB3681 at baseline, i.e at day zero, were quantitated by determining their MICs. However, routine MIC determinations and the techniques used in this study to elicit resistant mutants upon serial transfer in liquid culture containing sub-inhibitory drug concentrations differ from each other (micro- vs. macro dilution + incubation on a rotary shaker). Therefore, MICs of the test strains were determined in parallel by using first the standardised procedure as recommended by CLSI, and second by using the methods applied in this study.

To characterize the emergence of multistep drug resistance, bacteria were grown overnight in BHI broth containing 2-fold serial dilutions of MCB3681 (0.015 to 32 mg/l). From the culture containing the highest drug concentration permitting visible bacterial growth (i. e. 0.5 x MIC), an aliquot was transferred as a 1:20 dilution to inoculate a second set of serial antibiotic dilutions. After overnight incubation, the dilution procedure was repeated again over a total period of 7 days (1).
The bacteria were incubated on a rotary shaker at 37°C in 50 ml Erlenmeyer flasks in a final volume of 10 ml of medium.

RESULTS

I. Determination of MIC values (Table 1)

Table 1

Comparative determinations of MICs by using three different methods:
A) Microdilution acc. to DIN*
B) Macrodilution in test tubes incubated without agitation (10ml)
C) Macrodilution in 50 ml Erlenmeyer flasks containing 10ml of medium and incubated on a rotary shaker

Strains	A	B	C
Staph. aureus (MRSA) P8	0,06	0,06	0,125
Staph. aureus (MRSA) Visa Mu 50	0,125	0,25	0,5
Staph. aureus (MRSA) NRS 119	1	1	1
Staph. aureus (MRSA) ATCC 33593	0,06	0,125	0,5
Staph. aureus ATCC 29213	0,125	0,125	0,5
E. faecalis V 583	0,25	0,25	0,5
E. faecalis UW 3696	0,25	0,25	0,5
E. faecalis ATCC 29212	0,125	0,25	0,5
E. faecalis ATCC 51299	0,125	0,25	0,5
E. faecium BM 4147	0,25	0,25	0,5
E. faecium UW 3695	1	2	4
Strept. pneumoniae ATCC 49619	0,125	0,06	0,125
Strept. pneumoniae Bay 19397	0,125	0,125	0,125

* DIN: German Industry Guideline

As shown in table 1, the actual method used for determination of MIC value does have an influence on the outcome. Except for Streptococci, in all other cases the MIC value varied by a factor of 2 and even up to a factor of 8 when performed by agitated macrodilution. This has important influence on the determination of the frequency of resistance development as this is usually performed (and recommended by the CLSI guideline) at 4x the determined MIC value. E.g., an increase in the MIC value by a factor of 2 (one dilution step) results in the concomitant shift of the mutation frequency. This has to be put into perspective when presenting the absolute values of resistance frequencies and should always be shown to data from comparator antibiotics tested in the same laboratory.

II. Spontaneous emergence of resistance (Table 2).

Table 2: Emergence of singlestep resistance upon exposure to multiples of MICs of **MCB3681**

Emergence of single-step resistance influenced by MCB3681 (NG = no growth)

test strains	MIC (mg/l)	frequency of spontanous resistance
Staphylococcus aureus ATCC 29213 (MSSA)	0,125	
	2xMIC	$2,5 \times 10^{-7}$
	4xMIC	$4,8 \times 10^{-8}$
	8xMIC	ng
Staphylococcus aureus ATCC 33593 (MRSA)	0,06	
	2xMIC	$3,8 \times 10^{-7}$
	4xMIC	$7,9 \times 10^{-8}$
	8xMIC	$4,1 \times 10^{-8}$
Staphylococcus aureus NRS 119 (MRSA)	1	
	2xMIC	$7,3 \times 10^{-7}$
	4xMIC	$1,4 \times 10^{-7}$
	8xMIC	ng
Staphylococcus aureus Mu 50 (VISA)	0,125	
	2xMIC	$1,3 \times 10^{-7}$
	4xMIC	$1,8 \times 10^{-7}$
	8xMIC	ng
Staphylococcus aureus P - 8 (MRSA)	0,06	
	2xMIC	$6,1 \times 10^{-7}$
	4xMIC	$3,4 \times 10^{-7}$
	8xMIC	ng
Enterococcus faecalis ATCC 29212	0,25	
	2xMIC	$4,4 \times 10^{-8}$
	4xMIC	$1,2 \times 10^{-8}$
	8xMIC	ng
Enterococcus faecalis ATCC 51299 (VRE)	0,125	
	2xMIC	$1,2 \times 10^{-8}$
	4xMIC	$1,1 \times 10^{-7}$
	8xMIC	ng
Enterococcus faecalis V 583 (VRE,Van-B)	0,125	
	2xMIC	$2,3 \times 10^{-7}$
	4xMIC	ng
	8xMIC	ng
Enterococcus faecalis UW 3696 (LRE)	0,125	
	2xMIC	$3,2 \times 10^{-7}$
	4xMIC	1×10^{-7}
	8xMIC	ng
Enterococcus faecium BM 4147 (VRE, Van-A)	0,25	
	2xMIC	$9,7 \times 10^{-7}$
	4xMIC	$9,4 \times 10^{-8}$
	8xMIC	ng
Enterococcus faecium UW 3695 (LRE)	1	
	2xMIC	$3,7 \times 10^{-7}$
	4xMIC	ng
	8xMIC	ng
Streptococcus pneumoniae ATCC 49619	0,06	
	2xMIC	ng
	4xMIC	ng
	8xMIC	ng
Streptococcus pneumoniae Bay 19397 (FQ-resistant)	0,06	
	2xMIC	ng
	4xMIC	ng
	8xMIC	ng

2 X MICs
Exposure of bacteria to twice their MICs can almost be regarded as positive controls, as in general MIC determinations vary by plus/minus one titration step; thus, it is to be expected that the spontaneous mutation frequencies are high in the presence of 2x the individual MICs. Spontaneous mutation frequencies in the presence of 2x MICs vary from nil for the pneumococci, to 1.3×10^{-1} to 3.8×10^{-5} for the staphylococci and 2.3×10^{-5} to 1.2×10^{-6}, respectively, for the enterococci studied.

4 x MICs

- Staphylococci
  MSSA ATCC reference strain 29213 showed a very low frequency of 4.8×10^{-8}; colonies resistant to 4 x MIC of MCB3681 were detected only occasionally on the 50 plates examined. Higher single step resistance frequencies was only noted for the MRSA clinical isolate P8, it is not known whether this isolate is able to elicit this high resistance rate also towards other antibiotics by e.g. up regulation of a efflux pump or other non-target or non-compound related mechanisms.

- Enterococci
  No resistant subpopulations could be selected for strains E faecal is V583 (vanB) and E faecium UW 3695 (linezolid res.). The remainder strains showed low spontaneous mutation frequencies.

- Pneumococci
  Resistance to MCB3681 could neither be elicited in the fully-susceptible, nor in the penicillin- , macrolide- and ciprofloxacin resistant strain 19397.

8 X MICs
For only one strain resistant colonies could be elicited: MRSA ATCC 33593 = 4.1×10^{-8}; (colonies resistant to 8 x MIC of MCB3681 were detected only occasionally on the 50 plates examined).

Comparisons with other drugs
These data have to be put into perspective. Previous data generated in this laboratory under identical experimental conditions demonstrate that the mutational frequencies for other drug classes are comparably low for MCB3681 and 8-methoxyquinolones like e.g. moxifloxacin ($7.07x10^{-8}$ and $<1.45x10^{-9}$ at 4 X its MIC for MSSA and wild-type S pneumoniae, 3). Ciprofloxacin, however, is characterised by unfavourably high mutational frequencies against gram-positive bacteria (approx. $1x10^{-7}$ for MSSA and wild type S pneumoniae, 3). The mutational frequencies of amoxicillin, co-amoxiclavulanate, and cefuroxime at four times their MICs ranged from approx. 1×10^{-6} to 2×10^{-8}; those for the macrolide clarithromycin ranged from approx. 9×10^{-4} to 7×10^{-8} (4).

III. Emergence of multistep resistance (Table 3)

Table 3: Multistep emergence of resistance

Development of multistep resistance

Test strain	MIC [µg/ml]						
	day 1	day 2	day 3	day 4	day 5	day 6	day 7
Staph. aureus (MRSA) P 8 Day 0 = 0.125	0,25	0,125	0,25	0,5	0,5	1	1
Staph. aureus (MRSA) Visa MU 50 Day 0 = 0.5	0,5	0,5	1	2	2	4	4
Staph. aureus (MRSA) NRS 119 Day 0 = 1	2	2	4	4	8	16	16
Staph. aureus (MRSA) ATCC 33593 Day 0 = 0.5	0,5	0,25	1	0,5	1	1	1
Staph. aureus ATCC 29213 Day 0 = 0.5	0,5	0,25	0,5	1	2	8	8
Enterococcus faecalis V 583 Day 0 = 0.5	0,5	0,5	4	4	4	8	8
Enterococcus faecalis UW 3696 Day 0 = 0.5	0,5	0,25	0,5	0,5	1	0,5	0,5
Enterococcus faecalis ATCC 29212 Day 0 = 0.5	0,5	0,5	1	1	1	2	2
Enterococcus faecalis ATCC 51299 Day 0 = 0.5	1	2	2	4	4	8	8
Enterococcus faecium BM 4147 Day 0 = 0.5	0,5	0,5	2	2	4	8	8
Enterococcus faecium UW 3695 Day 0 = 4	4	1	2	4	4	4	4
Strept. pneumoniae ATCC 49619 Day 0 = 0.125	0,06	0,06	0,06	0,125	0,125	0,125	0,125
Strept. pneumoniae Bay 19397 MIC= 0.125	0,06	0,06	0,06	0,125	0,125	0,25	0,25

Staphylococci
The susceptibilities of all of the staphylococcal isolates tested except S. aureus NRS 119 remained unchanged from day 0 to day 2. From day 3 to day five, MICs increased by one to two dilution steps. A further increase in MICs was noted on days 6 and 7.

Enterococci
The MICs for all the enterococci (except those for the VRE reference strain ATCC 51299) remained unchanged from day zero to day two. Thereafter, the MICs increased by two- to three dilution steps from day 3 to day 5, followed by an additional one-fold increase of the MICs on day 6. The MICs of MCB3681 for the linezolid- and ciprofloxacin resistant isolates UW 3696 and UW 3695, resp., remained unchanged throughout the entire incubation period; likewise, the MICs for the susceptible reference strain ATCC 29212 increased marginally by one dilution step each from day 2 to 3 and from day 5 to 6. A moderate increase in MICs was noted for the vancomycin resistant reference strain ATCC 51299; the MICs for this strain increased by one dilution step each from days zero to 1, from day 1 to 2, from day 3 to 4 and again from day 5 to 6.

Pneumococci
Both pneumococcal isolates, the susceptible ATCC reference strain as well as the fluoroquinolone resistant strain No 19397 were negligibly affected by continuous exposure to sub-inhibitory MCB3681 concentrations over 7 days. The MICs increased by one to two dilution steps.

CONCLUSION

I. Spontaneous emergence of resistance

- In general, MCB3681 is characterised by a low propensity for resistance development in all the strains studied, including multidrug-resistant strains. No resistance at all could be elicited in the S. pneumoniae strains studied, irrespective of their penicillin-, macrolide-, or ciprofloxacin resistance. MCB3681 exhibited a low propensity for resistance development in E. faecalis and E. faecium, respectively, even in vancomycin- as well as linezolid resistant entrococcal strains.

- The spontaneous mutation frequencies for MCB3681 in methicillin susceptible and methicillin resistant reference-strain staphylococci as well as the VISA isolate were within the normal range, i.e. approx. 1×10^{-7} to 5×10^{-8}.

- Previous data generated in this laboratory under the same experimental conditions demonstrate that the singlestep mutational frequencies in S. aureus reference strains for other drug classes are comparably low for MCB3681 and 8-methoxyquinolones like e.g. moxifloxacin(3). Ciprofloxacin, amoxicillin, co-amoxiclavulanate, and cefuroxime at four times their MICs, however, are characterised by unfavourably high mutational frequencies against gram-positive bacteria (3, 4).

II. Multistep emergence of resistance

- Exposure of bacteria to sub-inhibitory concentrations may be clinically relevant for periods of 24h to 48h in incompliant patients (po administration), or in patients with altered drug metabolism or excretion (iv administration).

- Upon repeated exposure to sub-inhibitory concentrations of MCB3681 there was a moderate increase in MICs by ≥ 2 dilution steps up to day three in E. faecalis V 583 (vanB) and E. faecium BM 4147 (vanA).

- However, almost no shift (or an increase by one dilution step only) in susceptibilities to MCB3681 was noted in the remainder of the strains studied. In particular, this holds true for linezolid- and quinolone- as well as for vancomycin- resistant isolates. This finding is encouraging, first, as MCB3681 is a novel antibacterial agent in which an oxazolidinone-moiety was linked to a quinolone-moiety; second, resistance against these drugs rises continuously amongst the difficult to treat pathogens. Thus, MCB3681 can be characterized as an investigational drug with a low propensity for resistance development in selected difficult to treat gram-positive pathogens.

REFERENCES

1. Dalhoff, S. Schubert, U. Ullmann, Effect of pH on the in vitro activity of and Propensity for Emergence of Resistance to Fluoroquinolones, Macrolides, and a Ketolide Infection 2005; 33 (Suppl 2): 36-43
2. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, Approved Standard, NCCLS Vol. 20 No.2 M7-A5, Jan 2000
3. Dalhoff, U. Petersen, R. Endermann, In vitro activity of BAY12-8039, a new 8-methoxyquinolone. Chemotherapy 1996; 42: 410-425
4. S. Schubert, U.Ullmann, A. Dalhoff, Emergence of resistance in respiratory pathogens against faropenem in comparison to amoxicillin, amoxicillin-clavulanate, cefuroxime, and clarithromy-cin. Int J Antimicrob Agents 2001; 17 (Suppl 1): S89