Poster C1-1413
Session 169

Studies on the Mechanism of Resistance to PTK796 in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

A. Ruzin, J. Dzink-Fox, A.K. Jones, C.R. Dean, and P.A. Bradford — Department of Infectious Diseases, Novartis Institutes for BioMedical Research, Inc., Cambridge, MA, USA

Novartis Institutes for BioMedical Research, Inc.
500 Technology Square
Cambridge, MA 02139, USA
Phone: 617-871-5673 Fax: 617-871-4101
Email: alexey.ruzin@novartis.com

ABSTRACT

Background

PTK796 is a novel aminomethylcycline compound that is active against Gram-positive and Gram-negative bacteria. Certain Gram-negatives (e.g., *P. aeruginosa*) are intrinsically less susceptible to PTK796, whereas in others (e.g., *K. pneumoniae*), reduced susceptibility can be mutationally acquired. This study was conducted to provide initial insight into the mechanisms of resistance to PTK796 in *P. aeruginosa* and *K. pneumoniae*.

Methods

MICs were determined by broth microdilution using fresh media. Mutants were selected and spontaneous mutation frequency was measured on PTK796-containing plates. Gene expression was analyzed by Taqman RT-PCR.

Results

The MIC profile of *P. aeruginosa* efflux defective mutants revealed that deletion of the RND-type efflux pump *mexXY* reduced the MICs of PTK796 from 64 to 4 µg/mL. Inactivation of *mexB* had no effect; however, inactivation of *mexB* in the *mexXY* background caused an additional 4-fold increase in susceptibility. Less susceptible mutants (MIC 64-128 µg/mL) emerged at a frequency of 2.7×10^{-8} when the *mexXY*-deletion mutant was plated on 32 µg/mL of PTK796. The MICs of chloramphenicol and ciprofloxacin were elevated and sequence alterations were detected in *nfxB* gene (MexCD-OprJ repressor) in these mutants. In a less-susceptible strain of *K. pneumoniae* (MIC 16 µg/mL), the transcriptional activator *ramA* and the RND-type efflux pump *acrAB* were constitutively overexpressed as compared with a susceptible strain (MIC 1 µg/mL, typical for *K. pneumoniae*).

Conclusions

The results of this study suggested that: 1) similar to tigecycline, the inducible MexXY efflux pump is the major pump contributing to intrinsic decreased susceptibility in *P. aeruginosa*, with a smaller potential contribution from MexAB-OprM. In the absence of MexXY, MexCD-OprJ expressors emerge, indicating PTK796 is a substrate for this pump; 2) efflux is likely to be a primary determinant of acquired reduced susceptibility in *K. pneumoniae*, as indicated by overexpression of known tigecycline resistance determinants such as *ramA* and *acrAB*.

INTRODUCTION

PTK796 (7-dimethylamino, 9-(2,2-dimethyl-propyl)-aminomethylcycline) is a novel antibacterial agent that is active against both Gram-positive and Gram-negative bacteria.[1] Similar to tigecycline, the activity of PTK796 is not affected by classical tetracycline resistance mechanisms, including ribosomal protection and efflux by tetracycline-specific pumps.[2] Previous studies showed that decreased tigecycline susceptibility in Gram-negative bacteria is associated with constitutive overexpression of multidrug efflux pumps (MexXY, AcrAB, and AdeABC) that belong to the resistance nodulation division (RND) family.[3-8] In several species, e.g. *Klebsiella pneumoniae, Enterobacter cloacae,* and *E. coli,* efflux pump overexpression has been associated with constitutive overproduction of transcriptional activators such as RamA and MarA.[4,5,8,9] Certain Gram-negative bacteria (e.g., *P. aeruginosa*) are intrinsically less susceptible to PTK796, whereas in others (e.g., *K. pneumoniae*) reduced susceptibility can be mutationally acquired. This study was conducted to provide initial insight into the mechanisms of resistance to PTK796 in *P. aeruginosa* and *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

- The bacterial strains used in this study are shown in Table 1. The strains were propagated at 37°C in Luria–Bertani (LB) broth or agar

Table 1. Bacterial strains used in this study.

Species/strain	Description	Source or reference
P. aeruginosa		
K767	PAO1 parent strain	K. Poole
K1119	K767, *mexAB-oprM* and *mexXY-oprM* inactivated	K. Poole
K1523	K767, *mexB* deletion	K. Poole
K1525	K767, *mexXY* deletion	K. Poole
K1542	K767, *mexB* and *mexXY* deletion	K. Poole
NB52022-AVR0001	spontaneous mutant of K1525	This study
NB52022-AVR0003	spontaneous mutant of K1525	This study
NB52022-AVR0004	spontaneous mutant of K1525	This study
NB52022-AVR0005	spontaneous mutant of K1525	This study
NB52022-AVR0006	spontaneous mutant of K1525	This study
K. pneumoniae		
ATCC 700603		ATCC
ATCC 43816		ATCC

ATCC, American Type Culture Collection.

Antibiotic Susceptibility Testing

- Tigecycline and PTK796 used in this study were obtained from Wyeth Pharmaceuticals, Inc., Philadelphia, PA and from Paratek Pharmaceuticals, Inc., Boston, MA, respectively. Chloramphenicol, gentamicin, carbenicillin were obtained from Sigma Chemical Co., St. Louis, MO. Ciprofloxacin was obtained from US Pharmacopeia, Rockville, MD. The minimum inhibitory concentrations (MICs) of various antibacterial agents were determined by standard broth microdilution tests.[10,11] Tests for tigecycline and PTK796 were performed using fresh Mueller-Hinton broth (<12h old)

Selection of Mutants

- Less susceptible mutants of *P. aeruginosa* were selected by plating K1525 (*mexXY*-deletion mutant) on freshly made LB agar plates containing 32 µg/mL of PTK796

DNA Techniques and RT-PCR

- Oligonucleotide primers and probes used for polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR) are shown in Table 2. To prepare the templates for PCR, bacterial cells were taken from overnight LB agar plates, resuspended in nuclease-free water and boiled for 5 min followed by centrifugation to remove cell debris. The nucleotide sequence of gel-purified PCR fragments was determined by GENEWIZ, Inc., South Plainfield, NJ. DNase-treated RNA templates were prepared from mid-log phase bacterial cultures by using the RNAeasy kit (Qiagen, Valencia, CA). RT-PCR was performed by using an iScript One-Step RT-PCR Kit for Probes (Bio-Rad, Hercules, CA) on the CFX96™ Real-Time PCR Detection System. RT-PCR and the quantification of expression of the target genes (*mexC* in *P. aeruginosa*, *ramA* and *acrA* in *K. pneumoniae*) relative to an endogenous reference (16S rRNA gene) were performed as described previously.[7,9] Each sample was tested in duplicate.

Table 2. Primers and fluorescent probes used for PCR and RT-PCR.

Gene(s)	Purpose	Sequence (5'→3') of: Forward primer	Reverse primer	Fluorescent probe[a]
P. aeruginosa				
rpsL	PCR	CGATCTTCTATTGCACG	GCCAAGTGCCATATCG	NA[b]
mexC	RT-PCR	TTTGCTGCAATAGGAAGGATCGG	ACCTCACCGGCAACACCATTT	TATGGCCATCGCGTTGGCGGGTTGT
16S rRNA	RT-PCR	TAACGAGCGCAACCCTTGTCTTA	TACCGACATTGTAGCACGTGTGT	ACTCTAAGGAGACTGCCGGTGACAAA
K. pneumoniae				
acrA	RT-PCR	GTCCGGCACGCTGGA	ATAGCGCGTAGCGTGATTGA	CGGATGTCACCGTCGATCAGACCAT
ramA	RT-PCR	GCATCAACCGCTGCGTATT	CGTTGCAGATGCCATTTCG	ATCCCTCGCCATGCCGGGTAT
16S rRNA	RT-PCR	TTGACGTTACCCGCAGAAGAA	GCTTGCACCCTCCGTATTACC	TACTCCTGCCAGCAGCCG

[a]Labeled with 6-carboxyfluorescein at the 5' end and with 6-carboxytetramethylrhodamine at the 3' end. [b]NA, not applicable.

RESULTS

- The MIC profile of efflux-deficient mutants derived from the PAO1 strain of *P. aeruginosa* is shown in Table 3. Deletion of *mexXY* (RND-type efflux pump MexXY-OprM) reduced the MICs of PTK796 from 64 to 4 µg/mL. Inactivation of *mexB* (MexAB-OprM) had no effect; however, inactivation of *mexB* in the *mexXY* background caused an additional 4-fold increase in susceptibility, suggesting that PTK796 is also a substrate of MexAB-OprM
- Less susceptible mutants (PTK796 MIC 64-128 µg/mL, Table 3) emerged at a frequency of 2.7×10^{-8} when the *mexXY*-deletion mutant was plated on 32 µg/mL of PTK796. The MICs of chloramphenicol and ciprofloxacin were elevated and sequence alterations were detected in the *nfxB* gene (MexCD-OprJ repressor) in these mutants (Table 4). The amino acid substitutions at positions Ala38, Leu40, and Arg42 that resulted in overexpression of the MexCD-OprJ pump were described previously[12-14]
- The expression of *mexC* gene in *P. aeruginosa* strains was analyzed by RT-PCR (Figure 1A). In all of the spontaneous mutants that are less susceptible to PTK796, *mexC* was overexpressed 100- to 400-fold as compared with a susceptible parental strain K1525 (*mexXY* deletion)
- Gene expression data for two *K. pneumoniae* strains are presented in Figures 1B and 1C. In a less-susceptible strain ATCC 700603 (PTK796 MIC 16 µg/mL), the transcriptional activator *ramA* and the RND-type efflux pump *acrAB* were constitutively overexpressed, 40-fold and 5-fold respectively, as compared with a susceptible strain ATCC 43816 (PTK796 MIC 1 µg/mL, typical for *K. pneumoniae*)

Figure 1. RT-PCR analyses of gene expression.
Total bacterial RNA was isolated from mid-log phase cultures. Gene expression was analyzed by Taqman RT-PCR. The normalized expression ratios are shown. The error bars represent the standard deviation for the mean of duplicate samples.

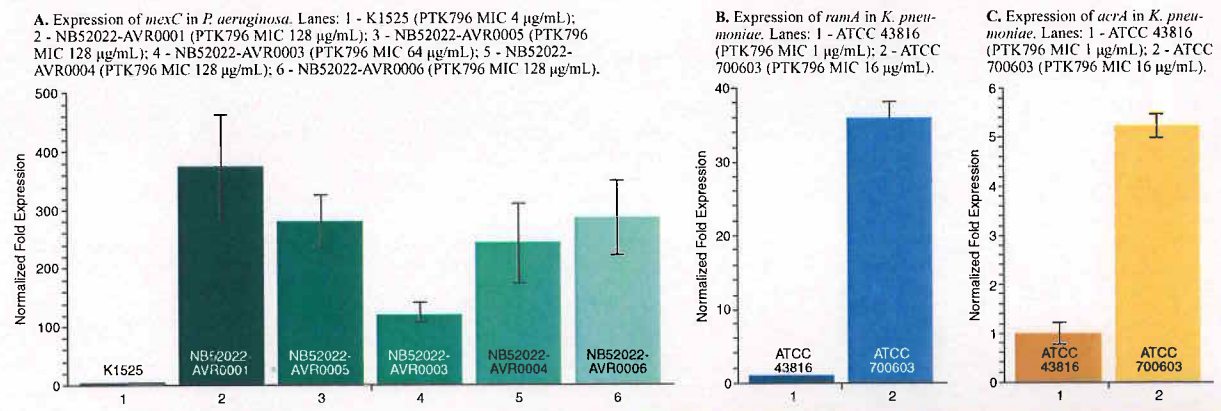

Table 3. Antibiotic susceptibility.

Strain	MIC (µg/mL) CHL	GEN	CIP	CAR	TGC	PTK
P. aeruginosa						
K767	128	2	0.125	256	8	64
K1119	4	0.25	0.015	ND	0.25	2
K1523	16	2	0.06	ND	8	64
K1525	256	0.25	0.125	256	0.5	4
K1542	8	0.25	0.03	ND	0.25	1
NB52022-AVR0001	512	0.06	4	64	8	128
NB52022-AVR0003	256	0.25	4	256	4	64
NB52022-AVR0004	512	0.125	4	32	8	128
NB52022-AVR0005	512	0.125	4	64	8	128
NB52022-AVR0006	512	0.125	4	64	8	128
K. pneumoniae						
ATCC 700603	32	4	0.5	>1024	2	16
ATCC 43816	4	0.25	0.03	64	0.125	1

Abbreviations: ATCC, American Type Culture Collection; CAR, carbenicillin; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; ND, not determined; PTK, PTK796; TGC, tigecycline.

Table 4. Alterations in *nfxB* gene in the spontaneous mutants that are less susceptible to PTK796 as compared with parental strain K1525.

Strain	*nfxB* mutation	Amino acid change
K1525	None	None
NB52022-AVR0001	ΔA_{71}-C_{71}	ΔMet1-Arg23 and frameshift
NB52022-AVR0003	$G_{370}T$	Gly180→Val
NB52022-AVR0004	$G_{124}A$	Arg42→His
NB52022-AVR0005	$C_{113}A$	Ala38→Asp
NB52022-AVR0006	$T_{119}A$	Leu40→Gln

CONCLUSIONS

- Similar to tigecycline, the inducible MexXY efflux pump appears to be the major pump contributing to intrinsic decreased susceptibility to PTK796 in *P. aeruginosa*, with a smaller potential contribution from MexAB-OprM
- In the absence of MexXY, MexCD-OprJ expressors emerge, indicating that PTK796 is a substrate for this pump
- Efflux is likely to be a primary determinant of acquired reduced susceptibility in *K. pneumoniae* as indicated by overexpression of known tigecycline resistance determinants such as *ramA* and *acrAB*. Gene knockouts are required to confirm the role of RamA and AcrAB in the decreased susceptibility to PTK796 in *K. pneumoniae*

ACKNOWLEDGMENTS

We thank Keith Poole for providing the *P. aeruginosa* strains.

REFERENCES

1. Macone, A., J. Donatelli, T. Dumont, S. B. Levy, and S. K. Tanaka. 2003. Presented at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, abstract F-254. 2. West, S., A. Macone, J. Donatelli, C. Tracy, D. E. Taylor, S. K. Tanaka, and S. B. Levy. 2003. Presented at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, abstract F-252. 3. Dean, C. R., M. A. Visalli, S. J. Projan, P. L. Sum, and P. A. Bradford. 2003. Efflux-mediated resistance to tigecycline (GAR-936) in *Pseudomonas aeruginosa* PAO1 strain. *Antimicrob Agents Chemother* 47:972-8. 4. Keeney, D., A. Ruzin, and P. A. Bradford. 2007. RamA, a transcriptional regulator, and AcrAB, an RND-type efflux pump, are associated with decreased susceptibility to tigecycline in *Enterobacter cloacae*. *Microb Drug Resist* 13:1-6. 5. Keeney, D., A. Ruzin, F. McAleese, E. Murphy, and P. A. Bradford. 2008. MarA-mediated overexpression of the AcrAB efflux pump results in decreased susceptibility to tigecycline in *Escherichia coli*. *J Antimicrob Chemother* 61:46-53. 6. Ruzin, A., D. Keeney, and P. A. Bradford. 2005. AcrAB efflux pump plays a role in decreased susceptibility to tigecycline in *Morganella morganii*. *Int J Antimicrob Agents Chemother* 26:791-3. 7. Ruzin, A., D. Keeney, and P. A. Bradford. 2007. AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. *J Antimicrob Chemother* 59:1001-4. 8. Ruzin, A., M. A. Visalli, D. Keeney, and P. A. Bradford. 2005. Influence of transcriptional activator RamA on expression of multidrug efflux pump AcrAB and tigecycline susceptibility in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 49:1017-22. 9. Ruzin, A., F. W. Immermann, and P. A. Bradford. 2008. Real-time PCR and statistical analyses of acrAB and ramA expression in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 52:3430-2. 10. CLSI. 2009. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M07-A8, 8th ed. vol. 29. Clinical and Laboratory Standards Institute, Wayne, PA. 11. CLSI. 2009. Performance standards for antimicrobial susceptibility testing. M100-S19. 19th informational supplement, vol. 29. Clinical and Laboratory Standards Institute, Wayne, PA. 12. Chuanchuen, R., K. Beinlich, T. T. Hoang, A. Becher, R. R. Karkhoff-Schweizer, and H. P. Schweizer. 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrob Agents Chemother* 45:428-32. 13. Jeannot, K., S. Elsen, T. Köhler, I. Attree, C. van Delden, and P. Plesiat. 2006. Resistance and virulence of *Pseudomonas aeruginosa* clinical strains overproducing the MexCD-OprJ efflux pump. *Antimicrob Agents Chemother* 50:2455-62. 14. Kaneyuki, E. S. Iofredi, B. Westfied, and D. I. Anderson. 2005. Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 55:22-30.