The Mechanism of Action of PTK 0796 (BAY 73-6944)

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ABSTRACT

Background PTK 0796 (7-dimethylamino, 9-(2,2-dimethyl-propyl)-aminomethylcycline) is a novel antibacterial agent of the tetracycline family with enhanced activity against gram-positive and gram-negative bacteria. To establish the mechanism of action, PTK 0796 was evaluated for its effects on *in vitro* macromolecular synthesis (protein, DNA, RNA, and peptidoglycan (pg)), ribosome binding, and an assessment of its killing activity was performed.

Methods Macromolecular synthesis in viable *S. aureus* was determined using radiolabeled substrates. Ribosome binding was determined by competition with radiolabeled tetracycline (tet) in *in vitro* binding assays. Bacterial killing was assessed by standard methods against several gram-positive organisms.

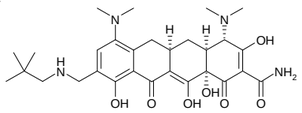
Results *In vitro* macromolecular synthesis assays demonstrated that PTK 0796 specifically inhibited protein synthesis, similar to tet. The IC50 was <0.03 µg/ml for protein synthesis, >32 µg/ml for RNA synthesis, >32 µg/ml for DNA synthesis, and 11.5 µg/ml for pg synthesis. PTK 0796 was a better competitor for ribosomal binding than unlabeled tet (IC50 2.32 µM vs 7.4 µM, respectively) in a competitive binding assay using [^{3}H]Tet. PTK 0796 exhibited an ability to kill *S. aureus* and *E. faecium* beginning within the first 2 hrs and extending through 24 hrs of incubation although not to the extent to be defined as a bactericidal agent.

Conclusions The antibacterial activity of PTK 0796 is linked to specific inhibition of bacterial protein synthesis. Competitive binding assays demonstrated that PTK 0796 effectively competed with tet for ribosome binding indicating that PTK 0796 shares ribosome-binding sites with tet and has greater affinity for these sites. In addition, PTK 0796 exhibited limited killing activity similar to that described previously for other tets.

INTRODUCTION

PTK 0796 (BAY 73-6944) is a novel aminomethylcycline that has excellent *in vitro* and *in vivo* activity against Gram-positive bacteria. These studies were conducted to establish the mechanism of action of PTK 0796 (BAY 73-6944) and how it compared with the standards, tetracycline and doxycycline.

STRUCTURE OF PTK 0796 (BAY 73-6944)



METHODS

Susceptibility Testing Susceptibility testing was performed according to the current NCCLS-recommended microdilution method. Cation-adjusted Mueller Hinton broth (MHB) was used. To prepare the inoculum, organisms were grown to a 0.5 McFarland standard which was measured with a Microscan turbidity meter. Microplates were incubated at 35ºC for 18 to 24 hours as specified by NCCLS.

Time-Kill Curves Time-kill curves were performed on tetracycline susceptible *Staphylococcus aureus* RN450 and clinical isolate *Enterococcus faecium* PBS838. An 0.8 ml aliquot of the cell suspension was added to 40 ml of media in flasks containing test compounds at chosen multiples of the MIC. Colony counts were used to determine the reduction in viable bacteria (log10 CFU/ml) at 0, 1, 2, 4, 6, and 24 hr.

Ribosomal Binding Ribosomes were collected from *E. coli* MRE600 on a sucrose gradient. A series of tubes were prepared with increasing concentrations (in µM) of the competitor test compound and a fixed concentration (3 µM) of radiolabeled tetracycline (^{3}H-Tc). 24 pmol of ribosomes was added to each reaction and incubated at 37°C for 5 min. The labeled tetracycline/test competitor solutions were added and the competition assay was incubated at 37°C for 40 min. The samples were collected on 25 mm nitrocellulose filters and washed 3 times. The filters were allowed to dry, added to a scintillation vial with scintillation fluid and CPM were measured.

Measure of *Tet*(K) Efflux on Total Protein Synthesis Macromolecular synthesis analysis was performed using two-fold dilutions of test compounds (ranging from 0.03 to 32 µg/ml) in a 96 well format. Overnight Mueller Hinton broth cultures of bacterial strains were diluted to an OD_{530} of 0.4 and incubated, shaking for 1 hr. These cells were used to inoculate a test plate containing diluted test and control compounds. Radiolabelled precursors were added and the plates were incubated, shaking at 37ºC. The reactions were quenched with 50% TCA and refrigerated for 1 hr before harvesting to a filtermat. The filtermat with scintillant was counted for 1 min. per sample in a Perkin Elmer Wallac (Boston, MA) Microbeta 1450.

RESULTS

Table 1. Bacterial Strains

Strains	Tet^R determinant	MICs		
		PTK 0796	tetracycline	doxycycline
S. aureus RN450	None	**0.125**	≤0.06	≤0.06
S. aureus ATCC 29213	None	**0.25**	0.125	≤0.06
S. aureus MRSA5	*Tet*(M)	**0.125**	>64	4
S. aureus RN4250	*Tet*(K)	**0.25**	32	4

The *in vitro* activity of PTK 0796, unlike that of tetracycline and doxycycline, was not affected by the presence of tetracycline resistance determinants.

Figure 1. Time Kill Curve, *S. aureus* RN450 vs. doxycycline

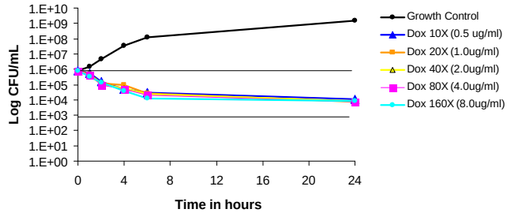
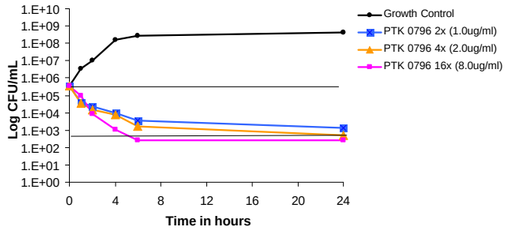


Figure 2. Time Kill Curve, PTK 0796 vs. *S. aureus* RN450



PTK 0796 exhibits improved early killing of *S. aureus* compared to doxycycline although neither achieves 3log10 reduction in viable counts at clinically relevant concentrations, within 24 hr.

Table 2. Effect of PTK 0796 on Macromolecular Synthesis in Susceptible Whole Cells of *S. aureus.*

Compound	Strain	IC50 (ug/ml)			
		Protein	RNA	DNA	PG
PTK 0796	**RN450**	**<0.03**	**>32**	**>32**	**11.6**
Tetracycline		0.04	31.4	25.7	8.8
Doxycycline		0.02	>32	>32	3.3
Rifampicin		0.01	0.01	>32	>32
Ciprofloxacin		14.0	>32	0.4	>32
Fosfomycin		>32	>32	>32	7.8
PTK 0796	**ATCC29213**	**0.19**	**>32**	**>32**	**15.7**
Tetracycline		0.09	23.7	>32	7.6
Doxycycline		0.08	>32	>32	2.9
Rifampicin		<0.01	0.01	>32	>32
Ciprofloxacin		>32	>32	0.3	>32
Fosfomycin		>32	>32	>32	11.9

PTK 0796 exhibits preferential inhibition of protein synthesis. In addition, PTK 0796 exhibits moderate inhibition of peptidoglycan synthesis, the significance of which is not known.

To further confirm that a new mechanism of action is not associated specifically in tetracycline resistant bacteria, the effect of PTK 0796 on macromolecular synthesis was determined in tetracycline resistant strains.

Table 3a. Effect of PTK 0796 on Protein Synthesis in Susceptible Compared to Resistant Whole Cells of *S. aureus*

Compound	Strain	TetR	MIC (ug/ml)	IC50 Protein Synthesis (ug/ml)
PTK 0796	**RN450**	**None**	**0.125**	**<0.03**
	ATCC29213	**None**	**0.25**	**0.19**
	RN4250	***Tet*(K)**	**0.25**	**0.08**
	MRSA5	***Tet*(M)**	**0.125**	**0.11**
Tetracycline	RN450	None	<0.06	0.04
	ATCC29213	None	0.125	0.09
	RN4250	*Tet*(K)	32	13.8
	MRSA5	*Tet*(M)	>64	1.8

Table 3b. Effect of PTK 0796 on RNA Synthesis in Susceptible Compared to Resistant Whole Cells of *S. aureus*.

Compound	Strain	TetR	MIC (ug/ml)	IC50 RNA Synthesis (ug/ml)
PTK 0796	**RN450**	**None**	**0.125**	**>32**
	ATCC29213	**None**	**0.25**	**>32**
	RN4250	***Tet*(K)**	**0.25**	**>32**
	MRSA5	***Tet*(M)**	**0.125**	**>32**
Tetracycline	RN450	None	<0.06	31.4
	ATCC29213	None	0.125	23.7
	RN4250	*Tet*(K)	32	>32
	MRSA5	*Tet*(M)	>64	>32

Table 3c. Effect of PTK 0796 on DNA Synthesis in Susceptible Compared to Resistant Whole Cells of *S. aureus*.

Compound	Strain	TetR	MIC (ug/ml)	IC50 DNA Synthesis (ug/ml)
PTK 0796	**RN450**	**None**	**0.125**	**>32**
	ATCC29213	**None**	**0.25**	**>32**
	RN4250	***Tet*(K)**	**0.25**	**>32**
	MRSA5	***Tet*(M)**	**0.125**	**>32**
Tetracycline	RN450	None	<0.06	25.7
	ATCC29213	None	0.125	>32
	RN4250	*Tet*(K)	32	>32
	MRSA5	*Tet*(M)	>64	>32

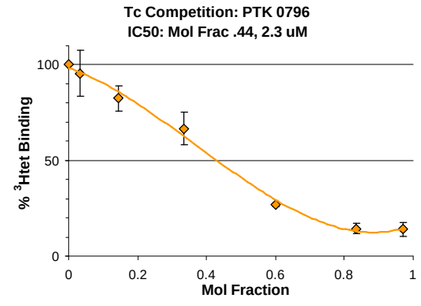
Table 3d. Effect of PTK 0796 on Peptidoglycan Synthesis in Susceptible Compared to Resistant Whole Cells of *S. aureus*.

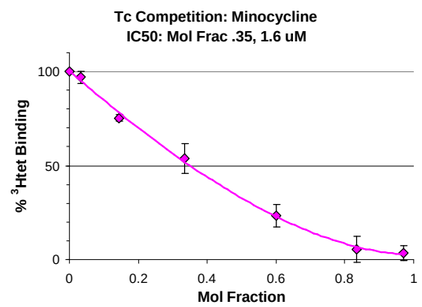
Compound	Strain	TetR	MIC (ug/ml)	IC50 PG Synthesis (ug/ml)
PTK 0796	**RN450**	**None**	**0.125**	**11.6**
	ATCC29213	**None**	**0.25**	**15.7**
	RN4250	***Tet*(K)**	**0.25**	**>32**
	MRSA5	***Tet*(M)**	**0.125**	**15.6**
Tetracycline	RN450	None	<0.06	8.8
	ATCC29213	None	0.125	7.6
	RN4250	*Tet*(K)	32	22.7
	MRSA5	*Tet*(M)	>64	>32

In contrast to tetracycline, the inhibitory activity of PTK 0796 against protein synthesis is not affected by the presence of either efflux (*tet*K) or ribosome protection (*tet*M), consistent with MIC determinations. Effects (or lack thereof) on other macromolecular synthetic processes were not related to *in vitro* susceptibility at relevant concentrations.

Figure 3. Competitive Ribosomal Binding for ^{3}H tetracycline

One additional method to confirm the classical tetracycline mechanism of action of PTK 0796 was to determine the ability of PTK 0796 to compete for the ribosomal binding site of tetracycline.





PTK 0796 competes for the ribosome binding site of tetracycline similar to minocycline. This finding confirms that PTK 0796 exerts its antibacterial activity by classically binding to bacterial ribosomes and inhibits protein synthesis.

CONCLUSIONS

PTK 0796 (BAY 73-6944), a novel aminomethylcycline,

- Exerts its antibacterial activity against tetracycline susceptible and resistant bacteria by inhibition of protein synthesis.
- In vitro MICs were not affected by tetracycline resistance mechanisms.
- Exhibited some bacterial killing activity.
- Protein synthesis was preferentially inhibited and at similar concentrations in the absence and presence of tetracycline resistance determinants.
- Binds to the tetracycline binding site of bacterial ribosomes.