

The Activity of PTK 0796 (BAY 73-6944) Against Tetracycline Resistance

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ABSTRACT

Background PTK 0796 (7-dimethylamino, 9-(2,2-dimethyl-propyl)-aminomethylcyclyne), a novel antibacterial agent of the tetracycline family, exhibits enhanced activity against gram-positive clinical isolates resistant to approved tetracyclines (tets). Studies were conducted to evaluate the potential emergence of resistance and the mechanism by which PTK 0796 circumvents tet resistance determinants.

Methods MIC testing was performed according to NCCLS guidelines. Tet resistance determinants (Tet(K), Tet(L), Tet(M)) were identified using multiplex PCR. Single-step mutant selection and passage studies were used to establish the likelihood of emergence of resistance. The influence of efflux was determined in Tet(K) *Staphylococcus aureus* and ribosome protection by *in vitro* translation with and without Tet(O).

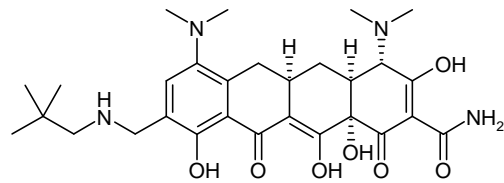
Results PTK 0796 has activity against both ribosomal protection and efflux mediated tet resistance. MICs were not significantly affected by the presence of Tet(M) (MIC range of 0.125 to 0.5 µg/ml), presence of Tet(L) or Tet(K) (0.125 to 0.25 µg/ml), compared to susceptible strains (<0.06 to 0.5 µg/ml). PTK 0796 exhibited no tendency to generate resistance in *S. aureus* with and without preexisting tet resistance either in single step selection or in serial passage. PTK 0796 does not block H-tet efflux and inhibits protein synthesis in the presence of Tet(O).

Conclusions PTK 0796 overcomes tet resistance mechanisms in gram-positive bacteria as shown by its potent activity against strains resistant to currently marketed tets. This activity was the result of PTK 0796 having low affinity for efflux pumps and greater affinity for ribosome binding than the protection protein. Regardless of the status of tet susceptibility of the test organisms, mutational resistance to PTK 0796 was not observed.

INTRODUCTION

Tetracycline resistance remains a clinically significant detriment to the utility of tetracycline, doxycycline, minocycline, and other commercially available tetracyclines. There are two major mechanisms of tetracycline resistance: efflux and ribosome protection. Both mechanisms have been described in gram positive and gram negative bacteria either separately or together, with the ribosome protection generally more common in gram positives and efflux in gram negatives. The most common genotypes of ribosome protection are *ter(M)* and *ter(O)*. Efflux is determined by a family of related genotypes in particular *ter(K)* and *ter(L)*. The goal of the Paratek scientists was to discover a novel class that overcomes both forms of resistance and as a result identified PTK 0796 (BAY 73-6944), a novel aminomethylcyclyne (AMC).

STRUCTURE OF PTK 0796 (BAY 73-6944)



METHODS

in vitro MICs

MICs were determined against recent clinical isolates using NCCLS methods.

Macromolecular Synthesis

Macromolecular synthesis analysis was used to establish the effectiveness of Tet(K), a tetracycline efflux protein against PTK 0796. Isogenic strains of *Staphylococcus aureus*, RN450 (*ter*^R) and RN4250 (*ter*^K, *ter*(K)), were used. The tests were performed using two-fold dilutions of test compounds (ranging from .03 to 32 µg/ml) in a 96 well format. Overnight Mueller Hinton broth cultures of bacterial strains were diluted to an OD₆₀₀ 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 37°C. The reactions were quenched with 50% TCA and refrigerated for 1 hr before harvesting to a filtermat. The filtermat with scintillant were counted for 1 min per sample in a Perkin Elmer Wallac (Boston, MA) Microbeta 1450.

In vitro Translation

Poly(U)-dependent Poly(Phe) synthesis (*in vitro* translation) was carried out using binding buffer conditions in 100 µl at 30°C for one hr. Tet(O) protein was added in a 1:1 molar ratio with ribosomes where included. Poly(Phe) chains were precipitated by adding 100 µl of 1% bovine serum albumin and 2 ml of 5% trichloroacetic acid (TCA) and incubated at 90°C for 15 min. The mixture was filtered through glass fiber filters, washed twice with 2 ml of 5% trichloroacetic acid and washed once with 2 ml of a 1:1 mixture of ether:ethanol. Filters were dried and counted after the addition of 300 ml of Soluene 350 (Packard, Meriden, CT, USA) and 7 ml of scintillation fluid.

Single-Step Resistance Selection

The single-step mutant analysis was performed in 100 mm square plates containing 40 ml of Brain Heart Infusion agar. A plate was prepared for each test concentration (4x, 8x, and 16x the MIC) of PTK 0796 and minocycline. The inoculum was no less than 1.5 X10⁸ CFU/plate. The plates were incubated at 37°C and examined for 3 days.

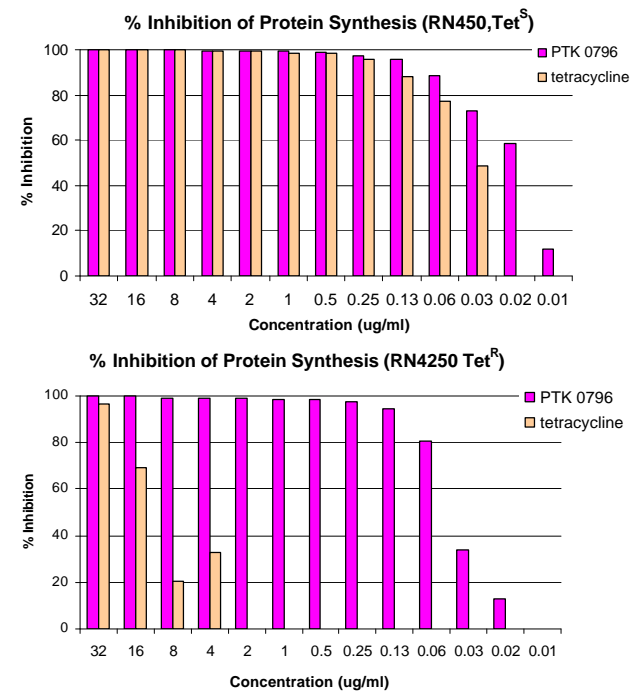
Multistep Resistance Selection

The passage study experiment involved 96 well microplates with 100 µl per well. The first test plate was inoculated with 5x10⁸ cfu/ml. Thereafter, the inoculum was taken from the well one dilution below the MIC well. One ul of this well was used to inoculate each well of a fresh MIC plate. This process was repeated for 10 days.

RESULTS

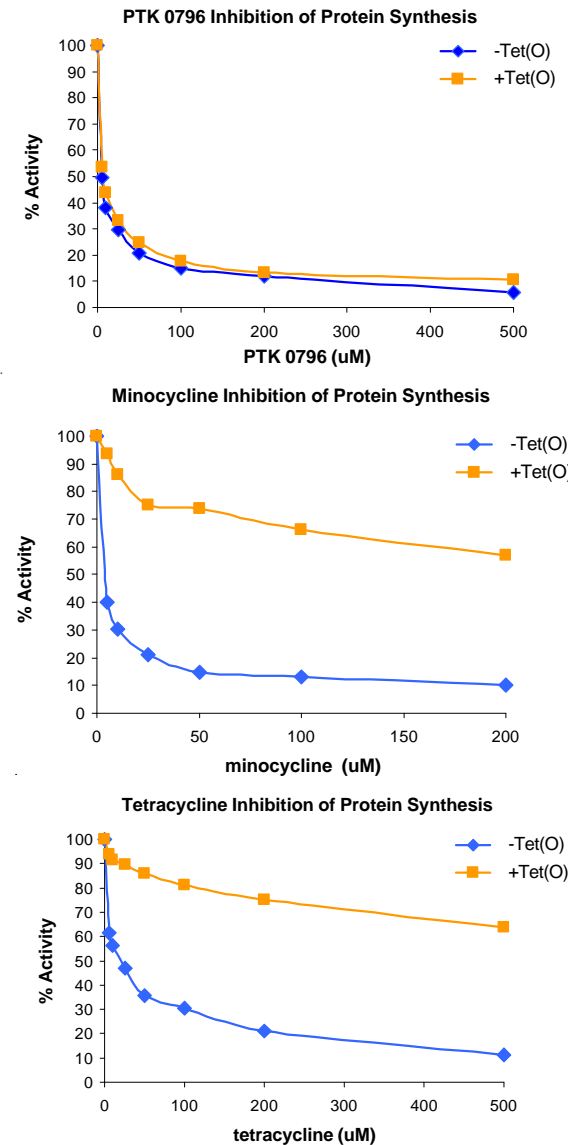
The ability of PTK 0796 to overcome tetracycline efflux was determined by comparing the effect of PTK 0796 on whole cell protein synthesis in the presence and absence of efflux.

Figure 1. Evaluation of the Effectiveness of Tetracycline Efflux in the Presence of PTK 0796 as Measured by Total Protein Synthesis



Unlike tetracycline, PTK 0796 effectively inhibits protein synthesis at low concentrations (IC50 0.01-0.04 mg/ml). Tetracycline is significantly affected by the presence of efflux *ter*(K) with the IC50 for protein synthesis increasing from 0.03mg/ml to approximately 12 mg/ml in the presence of efflux.

Figure 3. Effects of PTK 0796 on *in vitro* protein synthesis with and without the Ribosomal Protection Protein, Tet(O) in comparison to tetracyclines



PTK 0796 effectively inhibits protein synthesis in cell free systems whether the Tet(O) protein was present or not. Therefore, PTK 0796 binding to ribosomes was not affected by the presence of the ribosome protection protein. This result is in marked contrast to tetracycline and minocycline whose activity were inhibited by the presence of the ribosome protection protein.

Table 1. Activity of PTK 0796 Against Resistant Gram-Positive and Gram-Negative Aerobic Bacteria

Organism	Tetracycline Resistance Gene(s)	N	Compound		
			PTK 0796	MIC Range (µg/ml)	Doxycycline
<i>Staphylococcus aureus</i>	<i>ter</i> (M)	19	0.125 - 1.0	32 - >64	2 - 16
	<i>ter</i> (K)	5	0.125 - 0.25	16 - 32	1 - 4
	<i>ter</i> (L)	14	0.125 - 0.5	32 - 64	4 - 8
<i>Enterococcus faecalis</i>	<i>ter</i> (L)	1	0.25	64	16
	<i>ter</i> (M) and <i>ter</i> (L)	3	0.5	>64	16
	<i>ter</i> (S)	1	0.25	32	2
	<i>ter</i> (M)	13	0.125 - 0.5	32 - 64	2 - 8
<i>Enterococcus faecium</i>	<i>ter</i> (M) and <i>ter</i> (L)	2	0.25	>64	8 - 16
	<i>ter</i> (K)	1	0.12	32	4
	<i>ter</i> (O)	1	0.12	32	4
<i>Streptococcus pneumoniae</i>	<i>ter</i> (M)	22	<=0.06	4 - 64	2 - 4
	<i>ter</i> (M)	17	<=0.06 - 0.5	4 - 64	2 - 16
<i>β-hemolytic streptococci</i> (<i>S. pyogenes</i> , <i>S. agalactiae</i>)	<i>ter</i> (O)	4	<=0.06 - 0.25	32 - 64	8
	<i>ter</i> (B)	20	0.5 - 2	8 - 64	0.5 - 8
<i>Haemophilus influenzae</i>	<i>ter</i> (B) and <i>ter</i> (M)	2	1 - 2	16	2
	<i>ter</i> (A)	4	2	64 - >64	16

PTK 0796 was much more active against isolates with all forms of ribosome protection and tetracycline efflux than either tetracycline or doxycycline.

The ability to select PTK 0796 resistant mutants was determined in single and multiple passage experiments.

Table 2. Summary of Mutant Analysis Experiments

Strain	Tet ^R determinant	MICs		# Single Step Mutants at 4x, 8x or 16x MIC	Increase in MIC after 10 days of passage
		PTK 0796	minocycline		
<i>S. aureus</i> ATCC 29213	none	0.5	0.25	none	none
<i>S. aureus</i> MRSA5	<i>ter</i> (M)	0.5	4	none	none
<i>S. aureus</i> PBS468	<i>ter</i> (K)	0.5	0.25	none	none
<i>S. aureus</i> RN4250	<i>ter</i> (K)	0.5	0.5	none	none

Mutants resistant to PTK 0796 were not found either as single step mutants or multi-step mutants after up to 10 passages. Mutants were not observed whether the initial strain was susceptible or resistant to tetracycline (efflux or ribosome protection).

CONCLUSIONS

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

- Exhibited excellent activity against clinical bacterial isolates with a variety of tetracycline resistance traits.
- Did not appear to be recognized by the tetracycline efflux pump, Tet(K) when tested in whole cell protein synthesis assays comparing isogenic strains with and without Tet(K).
- In contrast to minocycline and tetracycline, overcame tetracycline resistance conferred by the ribosomal protection protein, Tet(O), in *in vitro* translation assays.
- Did not select single-step resistant mutants in the strains of *S. aureus* tested. This included strains carrying tetracycline resistance determinants *ter*(M) and *ter*(K).
- Did not select for multi-step resistant mutants in tetracycline sensitive and tetracycline resistant strains of *S. aureus* in a passage study over 10 days.

These results indicate that PTK 0796 (Bay 73-6944), has superior broad spectrum activity compared to tetracyclines, is not affected by tetracycline resistance, and exhibits a low propensity for resistance selection.