Poster 179
F1-2160
50th Annual ICAAC
12-15 September, 2010
Boston, MA

# Target- and Resistance-Based Mechanistic Studies with Fluorocyclines TP-434 and TP-271

A. L. STAROSTA[1], C. FYFE[2], W. O'BRIEN[2], D. N. WILSON[1], J. SUTCLIFFE[2], T. GROSSMAN[2]

[1]Univ. of Munich, Germany, [2]Tetraphase Pharmaceuticals, Inc., Watertown, MA, USA

Contact:
Leland Webster
Tetraphase Pharmaceuticals
lwebster@tphase.com

## Revised Abstract

**Background**: TP-434 and TP-271 are novel, broad-spectrum fluorocyclines with activity against major resistance mechanisms including tetracycline (Tet)-specific efflux and ribosome-protection.
**Method:** The activities of TP-434 and TP-271 were compared to those of tigecycline, PTK 0796 and Tet. Anti-translational activity ($IC_{50}$ values) was assessed in an *Escherichia coli in vitro* coupled transcription/translation assay (TnT) with a firefly luciferase readout. Purified Tet(M) (2-3 μM) was added to TnT reactions to evaluate ribosome protection effects *in vitro*. Binding of all compounds to empty ribosomes (0.4 μM *E. coli* 70S ribosomes) was measured in a competition assay with [$^3$H]Tet (8 μM). $IC_{50}$ values were determined ± increasing concentrations of competing compounds. Minimal inhibitory concentrations (MICs) were determined using standard CLSI methodology. Cell-based activity in the presence of Tet(M) (ribosome protection), Tet(A), Tet(B),Tet(K) (efflux) was evaluated in *E. coli* DH10B cells recombinantly expressing *tet*(M), *tet*(A), *tet*(B) and *tet*(K) under an L-arabinose inducible promoter. Antibacterial activity was evaluated against a set of *Propionibacterium acnes* mutants with target-based mutations in 16S rRNA.
**Results:** The antibacterial activities of TP-434, TP-271, tigecycline and PTK 0796 were unaffected, or minimally affected, by *tet*(M), *tet*(A), *tet*(B), and *tet*(K) expression in *E. coli;* however, PTK 0796 and Tet were significantly less potent against *E. coli* cells. TP-434 and TP-271 showed potent mechanism-based activity in the TnT and [$^3$H]Tet competition assays. The antibacterial potency of all tetracyclines was reduced by the 16S rRNA mutation G1058C in *P. acnes*. Taken together, the findings support a ribosome binding-mechanism of action for TP-434 and TP-271.
**Conclusion:** TP-434 and TP-271 are members of a new class of antibiotics whose anti-translation activity is unaffected by the common tetracycline-resistance mechanisms.

## Methods

***In vitro* coupled *E. coli* transcription/translation assay.** Anti-translational activity ($IC_{50}$ values) was assessed in an *E. coli in vitro* coupled transcription/translation assay (TnT) with a firefly luciferase readout (Promega, Madison, WI). Purified Tet(M) (2-3 μM) was added to TnT reactions to evaluate ribosome protection effects in vitro. Reactions were run at a volume of 22 μl in Costar black 96-well assay plates (Costar #3915) for one hour at 37ºC. The reaction was stopped by placing on ice for 5 minutes followed by addition of 25 μl/well of luciferase substrate (Promega, Madison, WI.). Plates were read on a LUMIStar Optima (BMG Labtech) with gain set to 3600, 0.2 second read, 0 seconds between wells. Percent luminescence was plotted against inhibitor concentration with 50% inhibition, versus untreated controls, marked as the $IC_{50}$ value. Results presented are an average of at least two independent experiments.

**[$^3$H]-tetracycline competition assay.** Binding of all compounds to empty ribosomes was examined using a competition assay with radiolabelled [$^3$H]tetracycline (Perkin Elmer) as described previously for erythromycin (Starosta et al., 2010). Briefly, all reactions contained 0.4 μM *E. coli* 70S ribosomes and 8 μM [$^3$H]tetracycline in binding buffer (10 mM HEPES/KOH pH 7.8, 30 mM $MgCl_2$, 150 mM $NH_4Cl$, 6 mM β-mercaptoethanol), which equated with 80% binding from the saturation curve (data not shown). To measure the $IC_{50}$ for each of the compounds, reactions were performed in the absence or presence of increasing concentrations of the competing compounds. After incubation at room temperature for 2 hours, reactions were passed through nitrocellulose filters, type HA, 0.45 μm pore size (Millipore). Filters were washed three times with binding buffer and radioactivity was determined using a scintillation counter in the presence of Filtersafe (Zinsser Analytic) scintillant. Results were graphed and $IC_{50}$ values were determined. Results presented are an average of at least two independent experiments.

**Antibacterial activity against *E. coli* DH10B recombinantly expressing tetracycline-resistance genes.** Genes encoding *tet*(A), *tet*(B), *tet*(K), *tet*(M), and E. coli β-galactosidase (*lacZ*) as a control were amplified by PCR from clinical isolates confirmed by prior sequencing to have these tetracycline-resistance determinants and cloned into an L-arabinose inducible expression system without any affinity tags (pBAD-Myc-His, Invitrogen, Carlsbad, CA). Plasmids were transformed and expressed in *E. coli* DH10B cells (Invitrogen, Carlsbad, CA). Cloned inserts were sequenced to verify the tetracycline resistance gene sequence and compared to reported sequences in GenBank (accession numbers; *tet*(A), AJ419171; *tet*(B), AP0961; *tet*(K), AJ888003; *tet*(M), X90939.1). Cells were grown in Mueller Hinton Broth containing ampicillin, 50 μg/ml, pre-induced for 30 minutes with 1% arabinose (*tet*(A), *tet*(B), *tet*(M)) or 0.1%arabinose (*tet*(K)) at 30ºC prior to use as inocula in MIC assays containing ampicillin, 50 μg/ml. Assays were incubated at 35ºC as per CLSI guidelines.

***Propionibacterium acnes* susceptibility testing.** Strains were obtained from the American Type Culture Collection or Stephen Shapiro (mutants described in Heller et al., 2007). *P. acnes* isolates were grown for three days at 37ºC on CDC Anaerobic Blood Agar Plates (BBL #221734) in a Mitsubishi 2.5 liter rectangular anaerobic box (Remel #R685025) using an anaerobic gas pack (Remel #R681001) with an anaerobic indicator strip (BBL #271051). Prior to assay, all strains were restreaked for isolation onto CDC anaerobic blood agar and grown for three days. Two-fold serial dilutions of test compounds were then prepared in a 96-well round bottom plate format in 50 μl Wilkens Chalgren Broth. Individual *P. acnes* colonies were picked and suspended in 2 ml 0.9% sterile saline to a turbidity equal to that of a 0.5 McFarland Standard (Remel #R6540). The bacterial suspension (100 μl) was aseptically transferred to 10 ml Wilkens Chalgren Broth and 50 μg/ml of organism was added to each well of the 96-well plate containing compound dilutions. Plates were incubated in an anaerobic box at 37ºC for 48 hours. MIC readings were then recorded following CLSI guidelines.

## Results

**Figure 1. Structures of TP-434 , TP-271 and comparators**

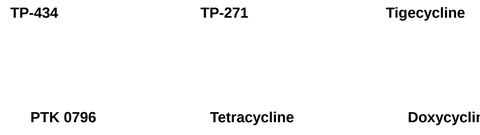


**Table 1. Summary of *in vitro* properties of TP-434, TP-271, and comparators**

| Assay | Antibiotic | | | | | |
|---|---|---|---|---|---|---|
| | TP-434 | TP-271 | Tigecycline | PTK 0796 | Tetracycline | Non-tetracycline |
| **TnT** $IC_{50}$, μg/ml | 0.29±0.09 | 0.18±0.08 | 0.08±0.01 | 0.66±0.06 | 1.26±0.48 | 1.30±0.28 (linezolid) |
| **TnT+TetM** $IC_{50}$, μg/ml | 0.27±0.16 | 0.13±0.04 | 0.09±0.04 | 0.43±0.16 | 6.5±3.3 | 1.08±0.74 (linezolid) |
| **[$^3$H]Tet Competition** $IC_{50}$, μM | 0.22±0.07 | 0.18±0.06 | 0.22±0.08 | 1.7±0.35 | 3.0±1.15 | >100 (erythromycin) |
| ***E. coli tet*(M)** MIC, μg/ml | 0.063 | 0.063 | 0.13 | 2 | 64 | 0.13 (ceftriaxone) |
| ***E. coli tet*(K)** MIC, μg/ml | 0.031 | 0.063 | 0.063 | 1 | 64 | 0.063 (ceftriaxone) |
| ***E. coli tet*(A)** MIC, μg/ml | 0.25 | 2 | 1 | 16 | >128 | 0.13 (ceftriaxone) |
| ***E. coli tet*(B)** MIC, μg/ml | 0.063 | 0.25 | 0.063 | 2 | >128 | 0.13 (ceftriaxone) |
| ***E. coli lacZ* (control)** MIC, μg/ml | 0.063 | 0.063 | 0.063 | 2 | 2 | 0.063 (ceftriaxone) |

**Figure 2. TP-434 and TP-271 compete with tetracycline for ribosome binding**

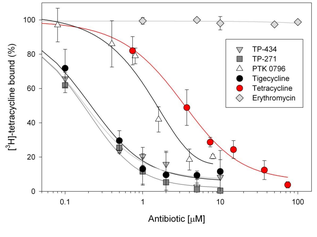


**Figure 3. TP-434 and TP-271 are unaffected by Tet(M) protein in an *in vitro* transcription/translation assay**

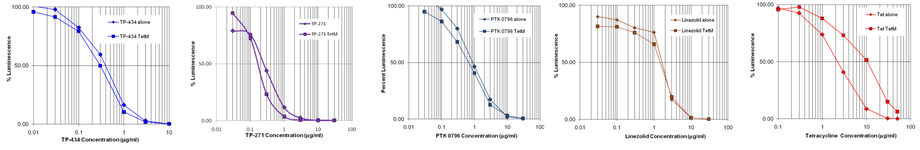


**Table 2. Activity of TP434, TP-271 and comparators against *Propionibacterium acnes* rRNA mutants**

| Strain Name: | ATCC 6919 | SW101 CDA | SW101T | P:413 |
|---|---|---|---|---|
| Genotype[1]: | wild type | *erm* (X) | 23S rRNA A2058G[3] | 16S rRNA G1058C |
| | | | 16S rRNA G1058C[3] | |
| Phenotype[2]: | | Ery-R, Clind-R, Tet-S | Ery-R, Clind-R, Tet-R | Ery-S, Clind-S, Tet-R |
| **TP-434** | 0.0625 | ≤0.0156 | 1 | 1 |
| **TP-271** | 0.0625 | ≤0.0156 | 2 | 1 |
| **Tigecycline** | 0.5 | 0.5 | 2 | 2 |
| **PTK 0796** | 2 | 0.5 | 32 | 32 |
| **Tetracycline** | 1 | 0.125 | 16 | 16 |
| **Doxycycline** | 1 | 0.125 | 8 | 8 |
| **Erythromycin** | 0.125 | >32 | >32 | 0.0625 |
| **Penicillin** | ≤0.0156 | ≤0.0156 | ≤0.0156 | ≤0.0156 |

[1] Genotype as described by Heller et al. 2007 AAC 51:1956-1961. 23S rRNA A2058G confers erythromycin/clindamycin resistance; 16srRNA G1058C confers tetracycline-resistance
[2] ery-R or ery-S, erythromycin-resistant or sensitive; clnd-R or clnd-S, clindamycin-resistant or sensitive; tet-R or tet-S, tetracycline-resistant or sensitive
[3]Base numbering based on *E. coli*

## Conclusions

- TP-434 and TP-271 are novel, potent, broad-spectrum, fluorocycline antibiotics targeting the ribosome
- [$^3$H]-tetracycline competition assay results and *P. acnes* mutant susceptibility data support that the binding sites of TP-434 and TP-271 at least partially overlap the tetracycline binding site
- TP-434 and TP-271 bound to empty ribosomes with ~14 and ~17-fold greater potency than tetracycline, and inhibited *in vitro* transcription/translation with ~4.3 and ~7-fold greater potency than tetracycline, respectively
- As compared to legacy tetracyclines the activities of TP-434 and TP-271 are:
  - Unaffected by ribosome protection of tetracycline resistance *in vitro* (TnT) and *in vivo* (*E. coli tet*(M) strain)
  - Minimally or unaffected by the most common tetracycline efflux pumps: *tet*(A), *tet*(B), and *tet*(K)

## References

**Starosta, A.L., Karpenko, V.V., Shishkina, A.V., Mikolajka, A., Sumbatyan, N.V., Schluenzen, F., Korshunova, G.A., Bogdanov, A.A., and Wilson, D.N.** 2010. Interplay between the ribosomal tunnel, nascent chain, and macrolides influences drug inhibition. Chem Biol *17*, 504-514.
**Heller, S., L. Kellenberger, S. Shapiro.** 2007. Antipropionibacterial activity of BAL19403, a novel macrolide antibiotic. Antimicrob Agents Chemother 51:1956-1961