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Novel Fully Synthetic Tetracyclines with Broad Spectrum Activity Against Biothreat Pathogens

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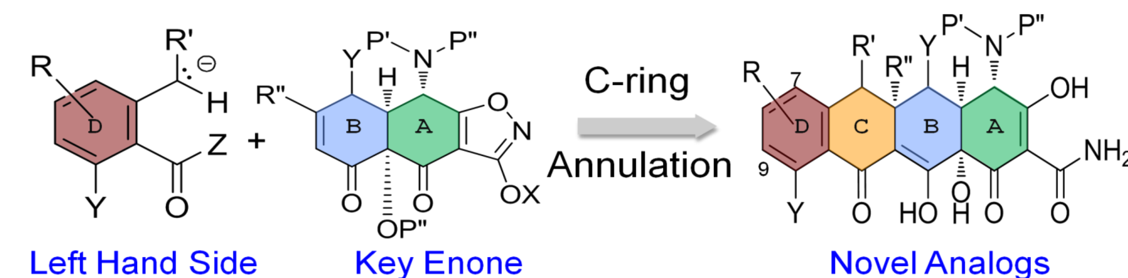
Abstract

Background. The threat of genetically engineered biological weapons requires the development of novel countermeasures with activity against drug-resistant isolates. The bacterial biothreats of greatest concern are *Bacillus anthracis, Francisella tularensis, Burkholderia* spp. and *Yersinia pestis*, all of which can be weaponized for use in an aerosolized mass attack. Preferred standard of care antibiotics such as aminoglycosides, fluoroquinolones, and tetracyclines could be rendered ineffective if pathogens were developed with resistance to these drugs. Tetracyclines remain an important antibiotic class distinguished by their clinically proven utility and safety in the treatment of respiratory infections caused by both common public health and biothreat pathogens. Notably, doxycycline remains a drug of choice used alone, and in combination, for many biothreat indications. The effectiveness of legacy tetracyclines has become limited by the dissemination of tetracycline-specific efflux and ribosome-protection mechanisms in gram-negative and gram-positive populations of public health pathogens. **Methods.** Using a proprietary, fully synthetic approach, 22 novel tetracycline derivatives were synthesized and screened against panels of ~30 isolates of each biothreat and individual clinical isolates of *Escherichia coli* and *Staphylococcus aureus* using standardized CLSI microtiter dilution assay methodology. Animal efficacy was tested in cytoxan-treated BALB/c mice infected intranasally with MRSA tet(M), dosed orally at 2 and 12 hrs post-infection, and colony forming units (CFUs) in lung were quantified at 24 hrs post-initial dose. **Results.** MIC_{90} values ranged from 0.015 -1 μg/ml for *Y. pestis*, 0.25 – 4 μg/ml for *F. tularensis*, $\leq$0.008 – 0.015 μg/ml for *B. anthracis*, 0.015 – 0.5 μg/ml for *B. mallei* and 2 – 16 μg/ml for *B. pseudomallei*. Further, many compounds were shown to retain activity (MIC <2 μg/ml) against *Escherichia coli* and *Staphylococcus aureus* isolates expressing tetracycline efflux (*tet*(A), *tet*(B), *tet*(K)) and ribosome protection (*tet*(M)) mechanisms. TP-271 and TP-4622, representative compounds of two distinct structural classes, each produced ~2-$\log_{10}$ reductions in bacterial burden in lung, versus the untreated control group, demonstrating the distribution of antimicrobially active compound into lung tissue. **Conclusion.** These findings support the promise of this synthetic tetracycline chemistry platform for the generation of novel oral countermeasures capable of protecting both the Warfighter and the public against an aerosolized biothreat attack.

Background

- The total synthetic approach discovered in Andrew Myers' lab (Charest, MG, et al. *Science* **2005**, *308*, 395-398), and applied to drug discovery by Tetraphase, has reinvigorated the field of tetracycline chemistry allowing for novel nuclei, including heterocyclic tetracyclines.



TP-271 is 7, 9 disubstituted
TP-4622 is 7, 8 disubstituted

- Small changes can profoundly affect spectrum, ability to evade resistance mechanisms, and pharmacokinetic properties

Methods

Susceptibility testing. All minimal inhibitory concentration (MIC) assays were performed as per CLSI guidelines. Testing of public health pathogens was performed at Tetraphase Pharmaceuticals using recent clinical isolates obtained from Eurofins Medinet. Testing of biothreat agents was performed at United States Army Medical Research Institute for Infectious Diseases (USAMRIID).

Animal infection models. All animal infection models were done at ViviSource Laboratories, Waltham, Massachusetts.

Neutropenic MRSA Lung model. Neutropenic BALB/c were made neutropenic with cyclophosphomide and challenged with a tetracycline-resistant *tet*(M) MRSA strain SA191 (n=6 per group). The MICs for TP-271, TP-4622 and linezolid were **0.25, 1.0 and 2** μg/mL, respectively. At 2 and 12 hours mice were dosed orally with 50 mg/kg compound and linezolid was dosed at 30 mg/kg. For IV administration, TP-271 and linezolid were dosed at 10 mg/kg at 2 and 12 hours. At 24 hours following initiation of treatment, mice were euthanized and bacterial reduction in the lung was quantified by plating lung homogenates.

***In vitro* transcription/translation assay.** Compound stocks prepared and diluted in sterile deionized water were assayed for inhibition of coupled *in vitro* transcription/translation using an *E. coli* S30 extract system with a firefly luciferase readout from Promega (Cat #L1020, Madison, WI). Briefly, compounds were diluted into water and added to reaction mix aliquoted to back-walled 96-well microtiter plates (Cat #3650, Costar, Corning, NY). An appropriate three-point titration was used for each compound, and reactions were run in duplicate. The final total reaction volume was 20 μl. Plates were incubated at 37 °C for one hour and then placed on ice for 5 minutes to arrest transcription/translation. Luciferase substrate (25 μl, Promega Cat #E1500) was added to each well and luminescence was detected on a BMG LabTech LUMIstar-OPTIMA instrument. Positive assay control values, from reactions without inhibitor, were averaged per plate to determine percent inhibition of luciferase production. Results were plotted using Microsoft Excel and fifty percent inhibition values (IC50) were determined.

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 30°C prior to use as inocula in MIC assays containing ampicillin, 50 μg/ml. Assays were incubated at 35°C as per CLSI guidelines.

Results

Figure 1. Activities of novel tetracyclines against biothreat pathogens

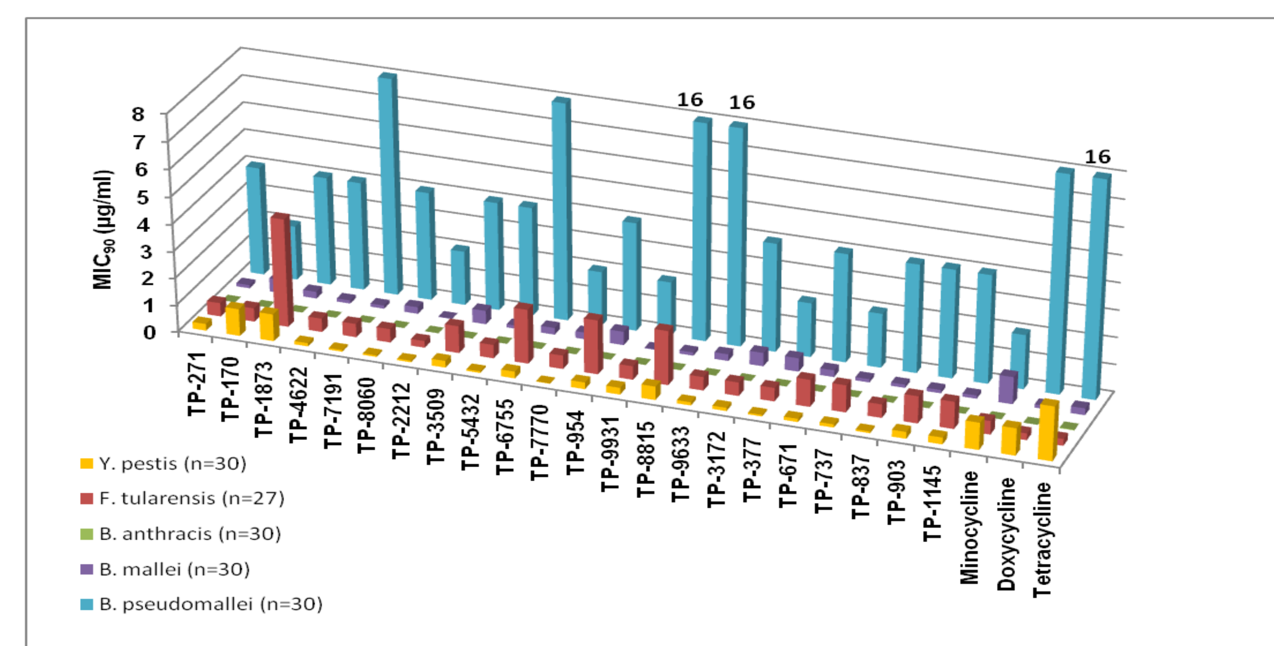


Figure 2. Activities of novel tetracyclines against tetracycline-resistant public health pathogens

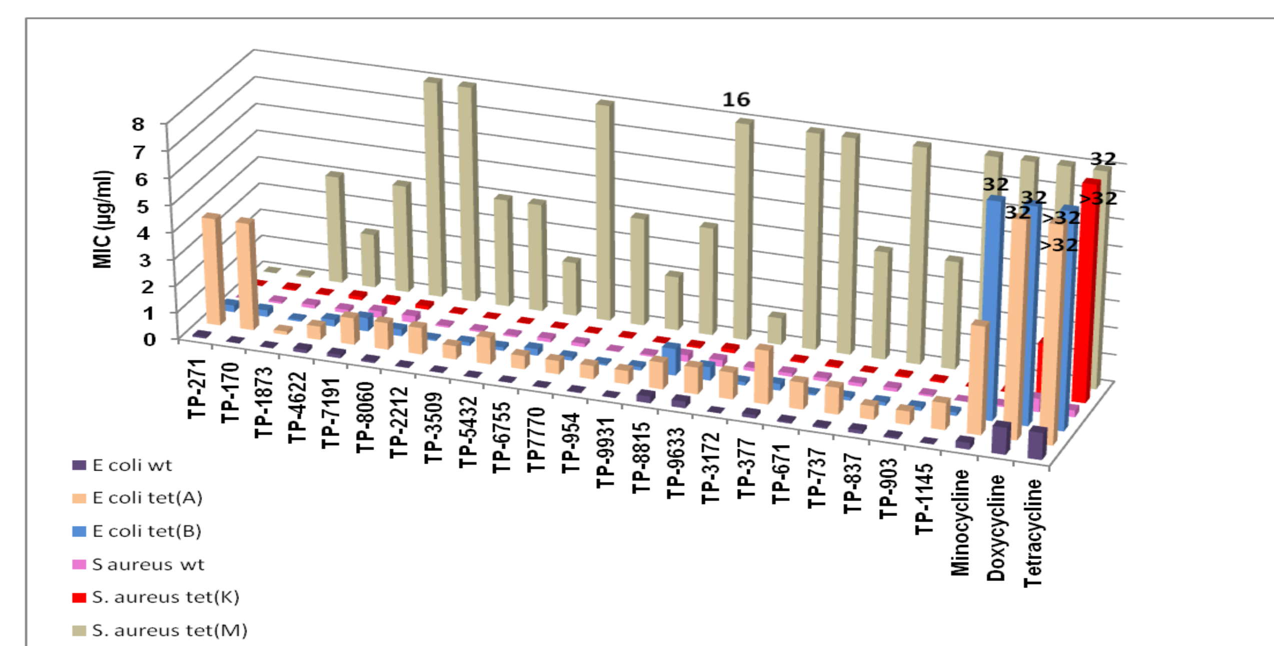


Table 1. TP-271 and TP-4622 are active against multidrug-resistant (MDR) public health pathogens

Organism	N	TP-271 MIC_{50}/ MIC_{90}	TP-271 Range	TP-4622 MIC_{50}/ MIC_{90}	TP-4622 Range	Erythromycin MIC_{50}/ MIC_{90}	Erythromycin Range	Doxycycline MIC_{50}/ MIC_{90}	Doxycycline Range	Levofloxacin MIC_{50}/ MIC_{90}	Levofloxacin Range	Resistant phenotype*
		Antibiotics (μg/ml)										
***S. aureus* (MRSA)**	38	0.031 / 0.25	$\leq$0.016-1	0.13 / 2	0.063-4	>32 / >32	0.25 - >64	0.25 / 16	0.063 - 16	8 / >32	0.13 – >32	tet, ery, levo, clinda, linez
S. pneumoniae	30	$\leq$0.016 / $\leq$0.016	$\leq$0.016- $\leq$0.016	0.031 / 0.5	$\leq$0.016-1	16 / >32	$\leq$0.016- >32	0.5 / 16	$\leq$0.016-16	1 / 1	0.5 – 1	tet, pen, ery, clinda,
H. influenzae	14	0.13 / 0.25	0.016-0.25	0.25/ 0.5	0.063-1	nd	nd	1 / 4	0.25-4	$\leq$0.016/ 0.031	$\leq$0.016-0.13	tet, amp
M. catarrhalis	14	$\leq$0.016/ 0.031	$\leq$0.016-0.063	0.063/ 0.063	0.063-0.13	0.25 / 0.5	0.13-2	0.13 / 8	0.13-8	0.031/ 0.063	0.031-0.063	tet, amp
E. coli	14	0.5 / 2	0.13 – 4	0.25 / 0.5	0.031-0.5	nd	nd	16 / >32	4 – >32	32 / >32	0.031 - >32	tet, levo, ESβL+, trim, gent, amp
K. pneumoniae	32	1 / 4	0.5 – >32	1 / 2	0.5 – 8	nd	nd	8 / 32	2 – 32	>32 / >32	0.06->64	tet, levo, ESβL+, carba, trim, amp, gent
A. baumannii	24	0.25 / 1	$\leq$0.016-2	0.25 / 2	$\leq$0.016-4	nd	nd	2 / >32	0.031 - >32	8 / 32	0.13 - >32	tet, tig, carba, levo, gent

***tet, tetracycline; tig, tigecycline; ery, erythromycin; levo, levofloxacin; pen, penicillin; clinda, clindamycin; amp, ampicillin; carba, carbapenem; gent, gentamicin; linez, linezolid; ESβL+, extended spectrum β-lactamase expression; trim, trimethoprim; nd, not done**

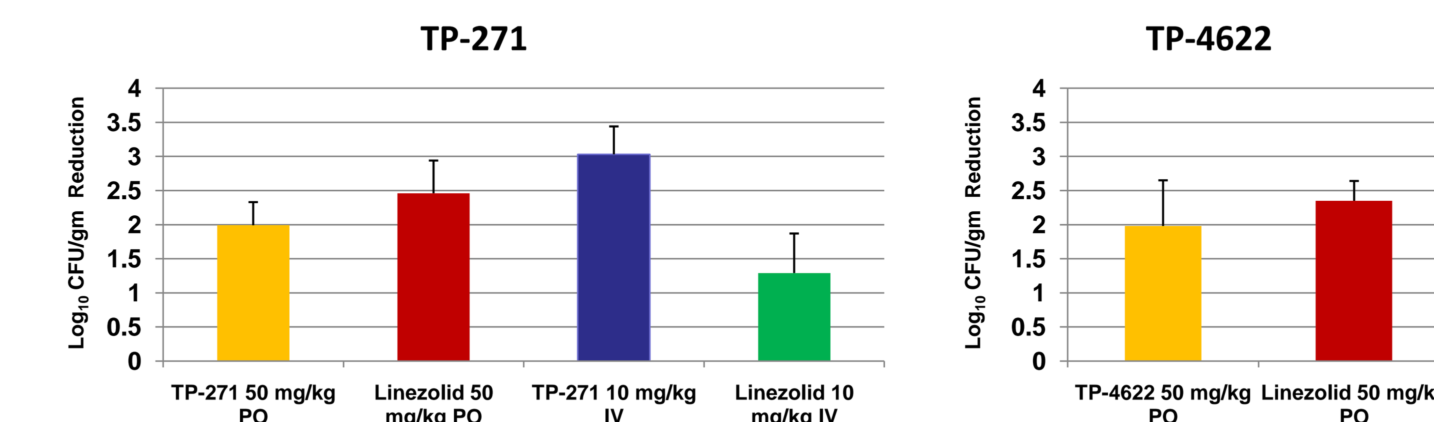
Results

Table 2. Novel tetracyclines are potent inhibitors of bacterial translation and retain activity against tetracycline-specific resistance

Assay	Compound: TP-271	TP-4622	Doxycycline	Tetracycline	Non-tetracycline
TnT IC_{50}, μg/ml	0.41 ± 0.40	0.40 ± 0.33	1.75 ± 0.64	1.67 ± 0.89	1.30 ± 0.28 *
E. coli tet(M) MIC, μg/ml	0.063	2	32	64	0.13 **
E. coli tet(K) MIC, μg/ml	0.063	0.063	8	64	0.063 **
E. coli tet(A) MIC, μg/ml	2	0.5	32	>128	0.13 **
E. coli tet(B) MIC, μg/ml	0.25	0.063	32	>128	0.13 **
E. coli lacZ (control) MIC, μg/ml	0.063	0.13	2	2	0.063 **

* data for linezolid
** data for ceftriaxone

Figure 4. TP-271 and TP-4622 are active in murine MRSA respiratory infection models



Conclusions

- Tetraphase's unique chemistry platform has enabled the complete synthesis of novel tetracyclines with potent broad-spectrum activity against five important biothreat agents: *Y. pestis, B. anthracis, F. tularensis, B. mallei,* and *B. pseudomallei*
- The antibacterial activities of novel tetracyclines are minimally affected by both MDR and tetracycline-specific resistance mechanisms
- The Tetraphase chemistry platform has the potential to generate novel countermeasures for the treatment of serious respiratory infections caused by multidrug-resistant public health and biothreat pathogens