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An evolved oxazolidinone with selective potency against *Mycobacterium tuberculosis* and gram positive bacteria

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Abstract

Innovation of new antibacterials that are effective against strains that have developed resistance to existing drugs would strengthen our ability to treat and subsequently control spread of pathogenic bacteria. Increasing incidence of infections with drug resistant bacteria has become a common occurrence in recent times. We have developed an evolved oxazolidinone, T145, which inhibits growth of *Enterococcus faecalis*, *Staphylococcus aureus* and *Mycobacterium tuberculosis* (*Mtb*) with sub $\mu\text{g/ml}$ potencies that are potentially therapeutically valuable. The oxazolidinone is bactericidal against *Mtb* but bacteriostatic against *E. faecalis* and *S. aureus*. In addition to therapeutically valuable potency and bactericidal activity against *Mtb*, T145 minimizes selection of spontaneous resistant mutants, a trait that prolongs longevity of a drug in clinical use.

Graphical Abstract

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COMPETING INTERESTS

None to declare.

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Keywords

antimicrobials; oxazolidinone; *Mycobacterium tuberculosis*; *Staphylococcus aureus*; *E. faecalis*

In 2008, the Infectious Diseases Society of America (IDSA) made the following ominous declaration: “The ongoing explosion of antibiotic-resistant infections continues to plague global and US health care”. Meanwhile, an equally alarming decline has occurred in the research and development of antibacterials to deal with the threat [1].’ In 2009, IDSA sounded another alarm by stating ‘we remain concerned that the infrastructure for discovering and developing new antibacterials continues to stagnate, thereby risking the future pipeline [2].’ Of major concern are the community and health care associated infections with bacterial pathogens *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (commonly abbreviated as *ESKAPE*) that are increasingly becoming resistant to drugs available to treat them.

In 2012, we established a Taskforce to study Resistance Emergence and Antimicrobial development Technology (TREAT) to address the emerging challenge of declining innovation in antimicrobials. In this translational research program, new agents are developed, evaluated for antimicrobial activity and the data is used to iteratively modify each pharmacophore to improve its therapeutic potential. Recently our group described synthesis of a novel oxazolidinone T145 (Figure 1) [3]. Linezolid, an oxazolidinone, initially labeled as U-100766, is a Food and Drug Administration approved antibacterial that is used for treatment of a wide range of bacterial pathogens [4]. Oxazolidinones have a common 2-oxazolidone ring and inhibit initiation of protein synthesis by preventing formation of ribosome and N-formylmethionyl-tRNA complex [5]. Resistance to oxazolidinones generally results from mutation in 23S rRNA [6, 7] which supports its mechanism of action, or due to activity of efflux pumps [8]. Toxicities associated with long term use of linezolid has spawned interest in newer oxazolidinones that improve on safety profile without compromising potency [9–11]. The initial report on T145 only measured its antibacterial activity against *S. aureus*. Here, we have investigated the activity of our novel oxazolidinone [3] against *ESKAPE* pathogens and *Mycobacterium abscessus* (*Mab*), *Mycobacterium avium* (*Mav*) and *Mtb*, the pathogen that causes tuberculosis (TB). We have studied two rifampin mono-resistant strains in addition to its parent drug sensitive strain. Although the World Health Organization declared TB a global health priority more than 20 years ago, this year it increased its estimate of death from this disease to 1.5 million making it the number one bacterial infectious disease in the world [12]. Emergence of multiple- and extensively-drug resistant strains of *Mtb* in many regions of the world clearly demonstrates that our efforts to innovate new agents against this pathogen have been inadequate [13]. Another emerging crisis is the increasing incidence of drug resistance in *ESKAPE* pathogens, often in community and hospital settings. This has meant added risk of acquiring potentially incurable infections in health care settings [2].

Mtb H37Rv,[14] *Mtb* 115R, *Mtb* 124R, *Mab* ATCC 19977, *Mav* 104 (isolated from an adult AIDS patient in Southern California in 1983), *A. baumannii* 6M-1b (an environmental

isolate), *A. baumannii* ATCC 19606, *A. baumannii* ACBA (isolated from patient at the Johns Hopkins Hospital) *E. faecalis* ATCC 19433, methicillin-sensitive *S. aureus* ATCC 29213, *E. cloacae* ATCC 13047, *K. pneumoniae* ATCC 35657 and *P. aeruginosa* PA14 were used. *A. baumannii* 6M-1b was recovered from Monocacy River near Frederick, MD, which is part of the upper watershed of the Chesapeake Bay. It was speciated using a validated MALDI-TOF MS assay (Bruker MicroFlex LT mass spectrometer and Bruker Biotyper software and existing database (version 2.0)) [15]. The log confidence score was >1.9, which is in the acceptable range for identification to the species level. In addition, a biochemical chart was also done (API 20NE, BioMerieux) which identified the isolate as *A. baumannii*. The API 20 NE is used for identification of non-enteric gram negative rods. *Mtb* 115R and 124R are mono-rifampin resistant laboratory isolates (from Dr. Eric Nuermberger, Johns Hopkins University) [16]: the rifampin minimum inhibitory concentration for the two strains were assessed in this study using broth dilution assay and were determined to be 4.0 and 128 µg/ml, respectively. *Mtb* H37Rv is the parent strain for 115R and 124R. *Mtb*, *Mav* and *Mab* were grown in Middlebrook 7H9 broth (Difco), containing 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC) and 0.05% Tween 80 or on Middlebrook 7H10 agar, 37 °C. *A. baumannii*, *E. faecalis*, *S. aureus*, *E. cloacae*, *K. pneumoniae* and *P. aeruginosa* were grown in cation-adjusted Mueller-Hinton broth (Becton-Dickinson). Liquid cultures were grown with constant shaking, 220 RPM, 37 °C. Meropenem, doripenem, faropenem, biapenem, tebipenem, clarithromycin and isoniazid were commercially acquired (Sigma-Aldrich). T145 was synthesized in the laboratory to a purity of >99% (Supplementary Figure 1).

Minimum inhibitory concentration (MIC₉₀) for *Mtb* was determined using standard broth macrodilution in 15-ml sterile conical tubes containing 2.5 ml of 7H9 broth. Standard broth microdilution method (using 96-well plates) was used for other organisms as per Clinical and Laboratory Standard Institute (CLSI) recommendations [17, 18]. Middlebrook 7H9 broth was used for *Mab*, *Mav* and *Mtb* and cation-adjusted Mueller-Hinton broth (Becton-Dickinson) was used for other organisms as per CLSI guidelines [18]. In summary, 10⁵ bacilli grown to exponential phase in liquid medium were inoculated into each well containing drug at two fold dilutions ranging from 64 µg/ml to 0.03 µg/ml. Growth medium alone and without drug but inoculated with 10⁵ bacilli were included as negative and positive controls, respectively. Appropriate drugs (isoniazid for *Mtb*, doripenem for *Mab*, clarithromycin for *Mav* and meropenem for *A. baumannii*, *E. faecalis*, *S. aureus*, *E. cloacae*, *K. pneumoniae* and *P. aeruginosa*) were included as positive control for growth inhibition. Growth was evaluated by visual inspection for presence of bacterial pellet following incubation (at 35 °C, 18 hours for *A. baumannii*, *E. faecalis*, methicillin-resistant *S. aureus*, *E. cloacae*, *K. pneumoniae* and *P. aeruginosa*, fourteen days for *Mtb* at 37 °C, 7 days, 37 °C for *Mav* and at 30 °C, 3 days for *Mab*) as per CLSI guidelines [18]. The first well in which bacterial pellet is absent and therefore growth is not observable is considered the MIC₉₀ as per the standard CLSI guidelines. MIC₉₀ is expressed as a range spanning two concentrations: the higher concentration represents the lowest concentration at which bacterial growth could not be observed.

Minimum bactericidal concentration (MBC_{99.9}), the minimum concentration of drug that kills 99.9% of bacilli, was determined by extending the broth microdilution assay described

above. Surviving bacilli or colony forming units (CFU) in wells in which growth could not be observed were determined by growing them on agar plates containing appropriate growth medium and enumerating CFU (after 2 days, 37 °C for *A. baumannii*, *E. faecalis*, *S. aureus* and *Mab* and 21 days for *Mtb*). The CFU at the initiation of the study, determined by enumerating live bacilli from initial inoculum on agar plates, is the input inoculum. The concentration of the agent at which 99.9% of the input CFU is killed is reported here as MBC_{99.9}. This assay is a modification of the broth dilution assay and was carried out as previously described [19]. In summary, each well containing 10⁵ CFU received two drugs each in two fold dilutions below their respective MIC₉₀. The suspensions are incubated at 37 °C and growth was evaluated as described above. Fractional Inhibitory Concentration (FIC) of a drug in a combination that inhibits bacterial growth is the concentration of the drug in the well divided by MIC₉₀ of the drug if used alone. The sum of FIC of each drug in the combination is the FIC index. In each well where bacterial growth was absent, FIC index was calculated and an average FIC index was determined. Drug-drug antagonism was inferred if average FIC index is >4, indifference if >0.5–4.0 and synergy if ≤ 0.5. Each organism was grown as described above to exponential phase, a suspension with optical density (A_{600nm}) of 1.0 was prepared and 1.0 ml of this suspension was spread onto each plate containing solid agar with growth media that was supplemented with T145 at 4–20× respective MIC₉₀. Five plates were used for each concentration of T145 studied. Colonies that grew at 37 °C (>2–3 days of incubation for *S. aureus* and at least 21 days for *Mtb*) were enumerated as spontaneous mutants resistant to T145. Colonies that grew on medium lacking T145 were enumerated as input CFU. The number of resistant mutants as a fraction of the input CFU inoculum was calculated as the frequency of spontaneous mutants resistant to T145.

Synthesis of T145 has been described in an earlier publication [3]. We synthesized a derivative of T145, labeled hereafter as T197 (Figure 2 and Supplementary Information). The rationale behind synthesizing and testing T197 was to determine the effect of adding an electron donating substituent to the benzodioxin ring. The methoxy group will also change the steric nature of the ring, which may affect its binding to its target(s). The synthetic route to T197 (an equal mixture of **9a** and **9b**) is shown in figure 2 and follows the same synthetic strategy used to synthesize T145 [3].

We assessed antimicrobial potencies of T145 and T197 against gram negatives *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. cloacae*, gram positives *E. faecalis*, and *S. aureus*, and acid fast pathogens *Mab*, *Mav* and *Mtb* by determining MIC₉₀ and MBC_{99.9}. Linezolid, an oxazolidinone that is clinically used for treatment of bacterial infections, was used as a control. Meropenem, one of the most potent antimicrobial available in clinic today [20], was also included as an additional control in this study.

T145 exhibited potent activity against growth of gram positive pathogens *S. aureus* and *E. faecalis* and *Mtb*, including two strains that exhibit low and high levels of resistance to rifampicin (Table 1). T145 displayed no activity against the gram negative pathogens *E. cloacae*, *K. pneumoniae* and *P. aeruginosa* and non-tuberculosis mycobacteria *Mab* and *Mav*.

We evaluated three axenic isolates of *A. baumannii* for their susceptibilities against T145 and T197. *A. baumannii* 6M-1b is an environmental strain; 19606 is an ATCC typed strain and ACBA is a recent clinical strain. The environmental strain 6M-1b displayed sensitivity to T145, T197, linezolid and meropenem with a MIC₉₀ of 0.25–4.0 (Table 1). Next, strain ATCC 19606 was marginally susceptible to T145 only (MIC₉₀=32–64 µg/ml). With an MIC₉₀ of >64 µg/ml for T145, T197, linezolid and meropenem, the patient isolate ACBA was most resistant among the three strains. The environmental strain of *A. baumannii* likely was not exposed to oxazolidinones and consequently was never under selective pressure of this agent. The patient isolate ACBA is likely to have the most exposure to drugs amongst the three strains. This data suggests that an increasing exposure to drugs has allowed *A. baumannii* to develop considerable resistance to evolved oxazolidinones T145 and T197.

It is noteworthy that potency of T145 is several fold superior to meropenem against *E. faecalis* and *Mtb*. The 0.5–1.0 µg/ml MIC₉₀ of T145 for *Mtb* H37Rv, and rifampicin mono-resistant strains 115R and 124R, is comparable to 0.5–2.0 µg/ml MIC₉₀ of linezolid, a clinically used oxazolidinone [4, 21]. Today linezolid is one of the alternate drugs used for treatment of multi- and extensively-resistant tuberculosis based on its potency to treat *Mtb* infections [22]. Clinical utility of T145 will be largely determined by its pharmacokinetic, pharmacodynamics and toxicity characteristics. T197, a derivative of T145, displayed antimicrobial activity against *A. baumannii* 6M-1b, *S. aureus*, *Enterococcus faecalis* and *Mtb*, but its potencies were several fold inferior to T145 (Table 1). It appears that the methoxy substituent that differentiates it from T145 decreases the antibacterial activity. It is possible that isomers 9a and 9b have different antibacterial activities but even if one had no activity and the other isomer all the activity, the one with all the activity would not be as active as T145. Determining which isomer is more active requires separation of the isomers and is currently being pursued. Also the position of the group on the ring will be varied in future work to determine if it is a steric or electronic effect causing this reduced activity.

To further investigate the antimicrobial potency of T145 we determined MBC_{99.9} for *Mtb* H37Rv, *E. faecalis* and *S. aureus*. For this, each pathogen was incubated in the presence of T145 at 1, 2, 4 and 8 times respective MIC₉₀ and the number of surviving bacilli were determined by enumerating CFU. T145 exhibited bactericidal activity against *Mtb* with MBC_{99.9} of 2 µg/ml. *E. faecalis* and *S. aureus* grew confluent on solid medium containing up to 8 times the MIC₉₀ of T145. Based on the lack of observable difference in CFU at and up to 8 times MIC₉₀, we conclude that T145 is bacteriostatic against *E. faecalis* and *S. aureus*. Linezolid also exhibits bacteriostatic activity against *S. aureus* [4].

As a therapeutic agent, a drug becomes ineffective for treatment of infections that develop resistance against it. Therefore, the frequency at which spontaneous mutation(s) that render a pathogen resistant to a drug arise is a measure of the longevity of the drug. We subjected *Mtb*, *E. faecalis* and *S. aureus* to T145 at 5×–60× their respective MIC₉₀ to determine the frequency of spontaneous resistant mutants. We were able to isolate *Mtb* mutants after 5 weeks of incubation at 5× and 10× MIC₉₀ of T145 and conclude that spontaneous mutations that confer resistance to T145 arise at a frequency of 4.0×10^{-8} . *E. faecalis* and *S. aureus* did not form classical distinct single colonies on agar plates containing T145. We consistently observed a thin semi-transparent layer atop the agar, which we determined was

the initial 1 ml culture deposited on it, on every plate that contained T145 at concentrations up to $60\times \text{MIC}_{90}$. Repeat attempts to select spontaneous mutants failed to produce distinct colonies. We hypothesize that T145 is bacteriostatic against *E. faecalis* and *S. aureus* at up to $60\times$ respective MIC_{90} and prevents selection of spontaneous resistant mutants. To test this hypothesis, we scraped the dried semi-transparent layer and transferred to fresh medium. The recovered organisms grew consistently in liquid broth lacking T145 but failed to grow in its presence at its MIC_{90} .

Carbapenems are a sub-class of β -lactams and derive their antimicrobial potency by inhibiting peptidoglycan biosynthesis [23]. We hypothesized that inhibition of two essential pathways, namely protein synthesis by T145 and peptidoglycan biosynthesis by carbapenems, may result in synergistic killing of bacterial pathogens. To evaluate if synergy (or indifference or antagonism) in antimicrobial activity exists between T145 and carbapenems, we used the checkerboard titration assay [19] and subjected *E. faecalis* and *S. aureus* to combination of the two agents, each at defined concentrations ranging from $1\times \text{MIC}_{90}$ to $1/16\times \text{MIC}_{90}$. Parenterally administered carbapenem biapenem, an orally bioavailable penam, faropenem and carbapenem tebipenem were studied as representatives of this class of β -lactams. Both *E. faecalis* and *S. aureus* are sensitive to these drugs (Table 2).

Growth inhibition of pathogens was not observed in any wells containing less than $\frac{1}{2} \text{MIC}_{90}$ of T145 or carbapenems demonstrating that antibacterial activity of T145 does not synergize with the tested carbapenems. Growth of the organisms was inhibited in wells containing T145 at $\frac{1}{2} \text{MIC}_{90}$. Based on these data we conclude that antimicrobial activity of T145 is indifferent (neither antagonistic nor synergistic) when combined with biapenem, faropenem and tebipenem. We also evaluated if any drug-drug interaction existed between T145 and isoniazid or rifampicin, two drugs that comprise the backbone of tuberculosis treatment today, against *Mtb* using the checkerboard assay as described above. We did not observe any antagonism or synergy between T145 and isoniazid or rifampicin, and conclude that activity of T145 against *Mtb* is indifferent to that of isoniazid or rifampicin.

Oxazolidinones are a relatively new class of antibacterials. Only two variants of oxazolidinones, namely linezolid and tedizolid are available for clinical use [25]. As most drug resistant strains of *Mtb* isolated from patients are generally sensitive to linezolid [26, 27], its off-label use to treat drug resistant TB is not uncommon [28–30]. The enthusiasm on oxazolidinones for treatment of *Mtb* infections, triggered by linezolid, has led to development of novel agents of this class. Multiple studies have concluded that toxicities associated with long term use of linezolid [9–11] warrant development of additional oxazolidinones that improve on safety without losing potency. Variants of linezolid have been described in recent years with improved potencies. Sutezolid (PNU-100480) is a more recent oxazolidinone with an *in vitro* potency against *Mtb* similar to linezolid [31]. Assessment using a murine model of TB demonstrated that sutezolid is more potent than linezolid and also possesses sterilizing activity, indicating the potential to shorten duration of therapy [32]. A recent study has described potent early bactericidal activity of sutezolid in tuberculosis patients [33]. AZD5847 is another experimental oxazolidinone with a reportedly improved *in vitro* potency against *Mtb* [34]. A direct comparison of linezolid,

sutezolid and AZD5847 demonstrated variable activities of the three oxazolidinones against *Mtb* [35].

While antibacterial potency and safety profile influence the potential for deployment of a new clinical agent, the frequency at which spontaneous resistant mutants are selected determines the longevity of the agent. A drug that has therapeutically superior potency, limited and tolerable toxicity and minimizes selection of spontaneous resistant mutant is likely to see effective deployment in the clinic. In addition, a simple and inexpensive synthesis of the drug would make it more accessible.

The frequency of *Mtb* spontaneous mutants resistant to T145 is similar to the frequency, 2×10^{-8} – 5×10^{-9} , described for linezolid [36]. With bactericidal activity against *Mtb* and *in vitro* potency that matches that of linezolid, T145 is a new oxazolidinone whose further evaluation of safety in clinical use has the potential to provide additional choice among this class of compounds for treatment of not only *Mtb* infections but also of gram positive pathogens. The potency of T145 against rifampicin resistant *Mtb* strains adds to its value as a potential agent for treatment of drug resistant TB. A robust program to develop new agent requires availability of multiple variants from which to optimize accessibility, potency, safety and frequency of resistant mutant selection. T145 provides that additional choice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

A collaboration between JTI and GL labs, AMH, JCC, DCE and JTI undertook chemistry studies and AK, DB and GL conducted activity assessments against bacterial pathogens. NK isolated and typed *Acinetobacter baumannii* strains 6M-1b and ACBA. This study was supported by a grant from Willowcroft Foundation and NIH award 1DP2OD008459 (GL) and institutional resources (JTI).

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HIGHLIGHTS

- Innovation of new antibacterials to treat drug resistant infections is necessary.
- Two evolved oxazolidinone were developed and their potencies were assessed.
- T145, inhibited growth of a range of bacterial pathogens with sub $\mu\text{g/ml}$ potencies.
- T145 is bactericidal against *M. tuberculosis* but bacteriostatic against *E. faecalis* and *S. aureus*.
- T145 minimizes selection of spontaneous resistant mutants.

Figure 1.
Structures of linezolid and T145.

Figure 2.

Synthetic route used to make T197. Reagents and conditions: a) K_2CO_3 , 80:30 DMF: toluene, reflux; b) H_2 , 10% Pd/C (50 bar, 50°C); c) TEA, THF, room temperature; d) THF, n -BuLi, $-78^\circ C$; e) methanesulfonyl chloride, TEA, DCM, $0^\circ C$; f) sodium azide, DMF, microwave $80^\circ C$ for 1h; g) H_2 , 10% Pd/C (30 bar, $30^\circ C$); h) acetic anhydride, pyridine, room temperature.

Table 1
Minimum inhibitory concentrations (MIC₉₀) of T145 and T197 in µg/ml

Data shown in this table were verified with two repeats of MIC determination. Meropenem and linezolid were used as controls, ND = not done.

Organism	MIC ₉₀ (µg/ml)			
	T145	T197	Linezolid	Meropenem
Gram negatives				
<i>Acinetobacter baumannii</i> 6M-1b	0.25–0.50	2–4	0.25–0.50	1–2
<i>Acinetobacter baumannii</i> ATCC 19606	32–64	>64	>64	>64
<i>Acinetobacter baumannii</i> ACBA	>64	>64	>64	>64
<i>Enterobacter cloacae</i>	>64	>64	>64	0.13–0.25
<i>Klebsiella pneumoniae</i>	>64	>64	16–32	<0.06
<i>Pseudomonas aeruginosa</i>	>64	>64	>64	0.25–0.50
Gram positives				
<i>Staphylococcus aureus</i>	0.5–1.0	16–32	1–2	0.06–0.13
<i>Enterococcus faecalis</i>	0.25–0.50	8–16	1–2	4–8
Mycobacteria				
<i>Mycobacterium tuberculosis</i> H37Rv	0.5–1.0	8–16	0.2–2.0 [4, 21]	4–8
<i>Mycobacterium tuberculosis</i> 115R	0.5–1.0	8–16	0.25–0.5	8–16
<i>Mycobacterium tuberculosis</i> 124R	0.5–1.0	4–8	0.25–0.5	8–16
<i>Mycobacterium abscessus</i>	>64	>64	ND	>64
<i>Mycobacterium avium</i>	>64	>64	ND	>64

Table 2

In vitro activity of doripenem, biapenem, faropenem and tebipenem against *Enterococcus faecalis*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*. Minimum inhibitory concentrations (MIC₉₀) in µg/ml are reported here.

	<i>Enterococcus faecalis</i>	<i>Staphylococcus aureus</i>	<i>Mycobacterium tuberculosis</i>
Biapenem	0.25–0.50	0.007–0.015	2.5–5.0 [24]
Faropenem	0.25–0.50	0.015–0.03	2.5–5.0 [24]
Tebipenem	0.25–0.50	0.03–0.06	1.25–2.5 [24]