Article

Synergistic Activity of Nitroimidazole-Oxazolidinone Conjugates against Anaerobic Bacteria

Zhijun Zhuang 1, Dawei Wan 1, Jun Ding 1, Shijie He 1, Qian Zhang 1, Xiaomei Wang 1, Ying Yuan 1, Yu Lu 2, Charles Z. Ding 3, Anthony Simon Lynch 4, Anna M. Upton 5, Christopher B. Cooper 5, William A. Denny 6, Zhenkun Ma 1,\*

1 TenNor Therapeutics Limited, 218 Xinghu Street, Building B2, Suite 711, Suzhou Industrial Park, Suzhou 215123, China; zhijun.zhuang@tennorx.com (Z.Z.); dawei.wan@tennorx.com (D.W.); jun.ding@tennorx.com (J.D.); shijie.he@tennorx.com (S.H.); qian.zhang@tennorx.com (Q.Z.); xiaomei.wang@tennorx.com (X.W.); ying.yuan@tennorx.com (Y.Y.)

2 Department of Pharmacology, Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing Chest Hospital, Capital Medical University, 97 Ma Chang Street, Beijing 101149, China; luyu4876@hotmail.com

3 Current address: WuXi AppTec. Co. Ltd., 288 Fute Zhong Road, Waigaoqiao Free Trade Zone, Shanghai 200131, China; charles\_ding@wuxiapptec.com

4 Current address: Janssen Research & Development LLC., 1400 McKean Road, Spring House, Pennsylvania 18940, USA; alynch2@its.jnj.com

5 Global Alliance for TB Drug Development, 40 Wall Street, New York, NY 10005, USA; anna.upton@tballiance.org (A.M.U.); christopher.cooper@tballiance.org (C.B.C.)

6 Auckland Cancer Society Research Centre, School of Medical Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand; b.denny@auckland.ac.nz

**\*** Correspondence: zhenkun.ma@tennorx.com; Tel.: +86-512-8686-1980

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**Abstract:** The introductions of the bicyclic 4-nitroimidazole and the oxazolidinone classes of antimicrobial agents represented the most significant advancements in the infectious disease area during the past two decades. Pretomanid, a bicyclic 4-nitroimidazole and linezolid, an oxazolidinone, are also part of a combination regimen approved recently by the US Food and Drug Administration for the treatment of pulmonary extensively drug resistant (XDR), treatment-intolerant or nonresponsive multidrug-resistant (MDR) *Mycobacterium tuberculosis* (TB). A series of dual-acting nitroimidazole-oxazolidinone conjugates was designed, synthesized and evaluated for their antimicrobial activity. Compounds in this conjugate series have shown synergistic activity against a panel of anaerobic bacteria, including those responsible for serious bacterial infections.

**Keywords:** Nitroimidazole-Oxazolidinone Conjugates, Anaerobic Bacterium, Synergy, Nitroimidazole and Oxazolidinone.

1. Introduction

Oxazolidinones, as represented by linezolid (**1**) and tedizolid (**2**), and bicyclic 4-nitroimidazoles, as represented by delamanid (**3**) and pretomanid (**4**), are two relatively new classes of antimicrobial agents (Figure 1). Linezolid, the first oxazolidinone approved for clinical use, was introduced in 2000 for the treatment of Gram-positive bacterial infections, including those resistant to other classes of antibiotics [1]. This drug class inhibits bacterial protein synthesis by binding to rRNA on both the 30S and 50S ribosomal subunits and preventing the formation of a translation initiation complex. The first bicyclic 4-nitroimidazole, delamanid, was introduced in 2014 for the treatment of drug-resistant tuberculosis (TB). This drug class utilizes a deazaflavin-dependent nitroreductase (Ddn) to catalyze the bioreduction of the 4-nitroimidazole core, leading to intracellular generation of reactive chemical species which are toxic to bacterial cells [2]. However, the primary mechanism of action of the bicyclic 4-nitroimidazole class against *M. tuberculosis* appeared to be different under aerobic and anaerobic conditions. Inhibition of mycolic acid synthesis appeared to be the main mechanism under aerobic conditions, while generation of reactive nitrogen species and inhibition of energy metabolism appeared to be the main mechanism under anaerobic conditions [3].

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**Figure 1.** Structures of Linezolid, Tedizolid, Pretomanid, Delamanid and Conjugate Molecule **5**

Resistance to oxazolidinones and bicyclic 4-nitroimidazoles are relatively uncommon. Both drug classes have been used for the treatment of multidrug-resistant (MDR) and extensively drug resistant (XDR) TB. Recently, a three-drug combination containing both pretomanid and linezolid has been approved by the US Food and Drug Administration for the treatment of pulmonary XDR, treatment-intolerant or nonresponsive MDR-TB [4].

Previously, we have utilized a drug conjugation strategy to identify the dual-acting molecule TNP-2092 for the treatment of bacterial biofilm infections [5]. This strategy provides several advantages as compared to a drug combination therapy, including matched pharmacokinetics, tissue distribution and potential synergistic activity. Considering the importance of the oxazolidinone and the bicyclic 4-nitroimidazole classes, a series of oxazolidinone-nitroimidazole conjugate molecules were designed, synthesized and evaluated against an isogenic mutant panel of *M. tuberculosis* conferring resistance to linezolid or pretomanid as well as a panel of clinically important anaerobic pathogens.

2. Results and Discussion

2.1. Chemistry

The safety and efficacy of the oxazolidinone and bicyclic 4-nitroimidazole classes have been validated clinically. The structure-activity relationships (SARs) of both drug classes have been extensively studied in the past, which serve as the foundation for the design of the conjugation molecules.

As a protein synthesis inhibitor, linezolid utilizes hydrogen bonding and hydrophobic interactions to bind to a binding domain located within the ribosomal peptidyltransferase center. Analysis of a high resolution of crystal structure of linezolid bound to the 50S ribosomal subunit indicates that the oxazolidinone ring and the acetamide group on the right side of the molecule are essential for the target interactions. The fluorophenyl moiety in the middle is also important. However, the morpholino ring on the left side does not appear to have significant interactions with the binding site, which is consistent with the known SARs that various structures can be used to substitute the morpholine group without a significant loss of activity [6]. This position was therefore identified as the linking point for conjugation to a bicyclic 4-nitroimidazole core.

On the bicyclic 4-nitroimidazole side, the nitroimidazole group and its fused oxazine or oxazole ring are essential and directly responsible for the formation of intracellular reactive species via a bioreduction process. The stereochemistry of the substituents connected to the oxazine or oxazole ring also plays an important role for the antimicrobial activity. However, the structure of the substituents on the right-hand side are highly variable, which can tolerate many functional groups. This site was hence identified as the linking point for the bicyclic 4-nitroimidazole class.

A series of drug conjugate were therefore designed and synthesized by connecting the right-hand side of the bicyclic 4-nitroimidazole and the left-hand side of the oxazolidinone core through various linkers. The syntheses of these conjugate molecules are illustrated in Scheme 1-3. Compound **5** (Figure 1) is a previously known oxazolidinone-metronidazole conjugate and it has not been evaluated for its activity against *M. tuberculosis* or anaerobic organisms [7]. Compound **5** was also prepared in the current study and evaluated for its activity against *M. tuberculosis* isogenic mutant panel and anaerobic bacterial panel.

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**Scheme 1.** Synthesis of Conjugate Molecules **8a-b** and **11a-b.**

Syntheses of the conjugate molecules formed by the oxazine fused nitroimidazole and the oxazolidinone core **8a-b** and **11a-b** are summarized in Scheme 1. The known (*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-ol core (**6**) was prepared according to literature procedures [8]. Intermediates **7a-b** and **10a-b** were prepared by aromatic or nucleophilic substitution reaction. Suzuki coupling with the oxazolidinone piece **9a** or **9b** provided conjugate molecules **8a-b** and **11a-b**.

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**Scheme 2.** Synthesis of Conjugate Molecules **14a-d** and **16.**

Syntheses of the conjugate molecules formed by the oxazole fused nitroimidazole and the oxazolidinone core **14a-d** and **16** are summarized in Scheme 2. The known 2-bromo-1-((2-methyloxiran-2-yl)methyl)-4-nitro-1*H*-imidazole (**12**) was prepared according to literature procedure [9]. Intermediate **13a-d** were prepared by epoxide opening followed subsequently by intramolecular cyclization. Suzuki or copper catalyzed coupling provided conjugate molecules **14a-d** and **16**.

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**Scheme 3.** Synthesis of Conjugate Molecules **19a-c.**

Scheme 3 summarizes the syntheses of a third series of conjugate molecules **19a-c**. This series contains the same oxazine fused nitroimidazole core as shown in Scheme 1 but connects to the oxazolidinones via a different linking point. The known intermediates (2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-7-yl)methanol (**17a**) and (7-methyl-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-7-yl)methanol (**17b**) were prepared according to literature procedure [10]. Aromatic substitution provided intermediates **18a-c**. Suzuki coupling with the oxazolidinone precursor **9a** provided conjugate molecules **19a-c**.

2.2. Mechanism of Action

The mechanisms of action of the precursor antibiotics of the conjugate molecules have been well established. In order to understand whether the conjugate molecules synthesized in the current study maintained antibacterial activity and received contribution from both precursor antibiotic classes, an isogenic panel of resistant mutant strains was prepared from wild-type strain *M. tuberculosis* H37Rv by stepwise resistance induction with linezolid or pretomanid (Table 1). Resistant mutant strains L1 and L3 were induced by linezolid with mutations on *rplC* gene encoding the 50S ribosomal protein L3. The minimum inhibitory concentrations (MICs) of linezolid against L1 and L3 were 4.80 and 8.18 µg/mL respectively, as compared to 0.15 µg/mL against the wild-type H37Rv strain. The resistant mutant strains P1 and P3 were induced by pretomanid with mutations in the *ddn* gene encoding the deazoflavin-dependent nitroreductase. The MICs of pretomanid against P1 and P3 strains were >20 and >20 µg/mL respectively, as compared to 0.07 µg/mL for the H37Rv strain.

All four conjugate compounds listed in Table 1 (**8a**, **8b**, **14c** and **14d)** were highly active against the H37Rv strain with MICs similar to that of linezolid and pretomanid. More importantly, all compounds were significantly more potent than linezolid against the L1 and L3 strains and significantly more potent than pretomanid against the P1 and P3 mutant strains, indicating that the antimicrobial activity of the conjugate molecules includes contributions from both parental antibiotic pharmacophores.

Compounds **8a** and **8b** were formed by the oxazine fused 4-nitroimidazole and the oxazolidinone core. These compounds, particularly compound **8a**, appeared to be more potent against linezolid resistant strains L1 and L3, suggesting that the 4-nitroimidazole function makes a relatively more significant contribution to the antibacterial activity than the oxazolidonone function in these conjugate molecules. However, compounds **14c** and **14d**, formed by the oxazole fused 4-nitroimidazole and the oxazolidinone core, demonstrated balanced activity against the linezolid resistant strains L1 and L3 and the pretomanid resistant strains P1 and P3, suggesting that these conjugate molecules exhibit a balanced contribution from the two parental antibiotic pharmacophores.

**Table 1.** Minimum inhibitory concentrations of selected conjugate molecules against isogenic resistant mutant strains of *M. tuberculosis*.

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| --- | --- | --- | --- | --- | --- |
| **Compounds** | ***M. tuberculosis* Isogenic Mutant Panel MIC (μg/mL)** | | | | |
| **H37Rv** | **L1** | **L3** | **P1** | **P3** |
| **Linezolid** | 0.15 | 4.80 | 8.18 | 0.29 | 0.31 |
| **Pretomanid** | 0.07 | 0.15 | 0.24 | >20 | >20 |
| **8a** | 0.06 | 1.95 | 1.78 | 6.48 | 5.96 |
| **8b** | 0.20 | 0.91 | 1.87 | 1.04 | 3.30 |
| **14c** | 0.08 | 1.84 | 0.81 | 1.77 | 1.80 |
| **14d** | 0.33 | 1.88 | 1.46 | 1.93 | 2.94 |

2.3. Spectrum of Activity

Selected compounds from the current study were tested against a panel of representative pathogens (Table 2). All eight conjugate compounds tested showed a similar spectrum of activity as linezolid against the ESKAPE pathogens: *Enterococcus faecium* (Ef), *Staphylococcus aureus* (Sa), *Klebsiella pneumoniae* (Kp), *Acinetobacter baumannii* (Ab), *Pseudomonas aeruginosa* (Pa), and *Escherichia* *coli* (Ec). Specifically, these compounds were active against Ef and Sa, but inactive against Kp, Ac, Pa and Ec strains. Several compounds (**8a**, **8b** and **11a**) were substantially more active than linezolid against Ef and Sa. All conjugate compounds were substantially more active than linezolid against the obligate anaerobic pathogen *Clostridium difficile* (Cd). The activities of these compounds were similar to that of metronidazole. These compounds were not cytotoxic with IC50s > 64 μg/mL for all compounds tested against the Vero cell-line.

**Table 2.** Minimum inhibitory concentrations of selected conjugate molecules against representative strains from seven major pathogen classes.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Compounds** | **Representative of Major Pathogens MIC (μg/mL)** | | | | | | | **Vero IC50 μg/mL** |
| **Ef**  **ATCC 708221** | **Sa**  **ATCC 29213** | **Kp ATCC 43816** | **Ab ATCC 19606** | **Pa ATCC 27853** | **Ec**  **ATCC 25922** | **Cd ATCC 43255** |
| **Linezolid** | 4 | 8 | >64 | >64 | >64 | >64 | 4 | >64 |
| **Levofloxacin** | >16 | 0.25 | 0.063 | 0.5 | 1 | <0.016 | 4 | - |
| **Metronidazole** | >64 | 64 | >64 | >64 | >64 | >64 | 0.25 | - |
| **8a** | 0.5 | 1 | >64 | >64 | >64 | >64 | 0.125 | >64 |
| **8b** | 2 | 2 | >64 | >64 | >64 | >64 | 0.25 | >64 |
| **11a** | 1 | 2 | >64 | >64 | >64 | >64 | 0.25 | - |
| **11b** | 4 | 8 | >64 | >64 | >64 | >64 | 0.125 | >64 |
| **14a** | 4 | 4 | >64 | >64 | >64 | >64 | 0.25 | >64 |
| **14b** | 8 | 16 | >64 | >64 | >64 | >64 | 0.5 | >64 |
| **14c** | 1 | 2 | >64 | >64 | >64 | >64 | 0.125 | >64 |
| **14d** | 4 | 4 | >64 | >64 | >64 | >64 | 0.5 | >64 |

2.4. Anaerobic Activity

The promising activity of the 4-nitromidazole-oxazolidinone conjugate series against the anaerobic pathogen *C. difficile* encouraged us to explore a broader panel of clinically important anaerobic bacteria (Table 3). The test panel included 13 strains of anaerobic bacteria and *Helicobacter pylori* (Hp) a gram-negative microaerophilic bacterium. Seven of the 13 anaerobic bacteria were Gram-positive and the rest were Gram-negative strains. The full names of the anaerobic bacterial panel strains are listed in Table 4.

**Table 3.** Minimum inhibitory concentrations of 12 conjugate molecules against clinically important anaerobic and microaerophilic bacterial strains

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Compounds** | **Anaerobic and Microaerophilic Bacterial Panel, MIC (ug/mL)** | | | | | | | | | | | | | |
| Gram-positive | | | | | | | Gram-negative | | | | | | Micro-  aerophilic |
| Cd  ATCC 700057 | La  ATCC 4356 | El  ATCC 43055 | Gv  ATCC 14018 | Pm  ATCC 23195 | Pas  ATCC 3553 | Pac  ATCC 11829 | Bf  ATCC 25285 | Bl  ATCC 15707 | Fn  ATCC 10953 | Mm  ATCC 35243 | Pv  ATCC 29303 | Vp  ATCC 17745 | Hp  ATCC  43504 |
| **Metronidazole** | ≤0.25 | >256 | 0.5 | 2 | >256 | 0.5 | >256 | ≤0.25 | 4 | ≤0.25 | 8 | 2 | >256 | 64 |
| **Pretomanid (P)** | 2 | >32 | 8 | >32 | >32 | 2 | >32 | 16 | >32 | 4 | >32 | 32 | >32 | 16 |
| **Linezolid (L)** | 0.5 | 2 | 1 | 0.25 | 1 | 0.5 | 0.06 | 2 | 0.5 | 0.25 | 0.12 | 2 | 0.5 | 8 |
| **L + P (1:1)** | 1 | 4 | 2 | 0.5 | 2 | 1 | 0.25 | 4 | 2 | 1 | 0.25 | 4 | 1 | 16 |
| **5** | 0.06 | NA | 0.06 | 0.12 | 0.25 | 0.06 | 1 | 0.25 | 0.5 | 0.06 | 0.25 | 0.5 | 1 | - |
| **8a** | ≤0.03 | 0.5 | ≤0.03 | 0.06 | 0.12 | 0.06 | ≤0.03 | 1 | 0.25 | ≤0.03 | ≤0.03 | 4 | 0.25 | 0.5 |
| **8b** | ≤0.03 | 0.5 | ≤0.03 | 0.06 | 0.25 | ≤0.03 | 0.06 | 4 | 0.12 | ≤0.03 | 0.06 | 8 | 0.25 | 1 |
| **11a** | 0.12 | 0.5 | 0.06 | 0.06 | 0.5 | 0.06 | 0.06 | 2 | 0.25 | 0.06 | 0.06 | 4 | 0.25 | 2 |
| **11b** | 0.06 | 2 | 0.06 | 0.12 | 1 | ≤0.03 | 0.12 | 4 | 0.25 | ≤0.03 | 0.25 | 8 | 1 | 2 |
| **14a** | 0.25 | 1 | 0.12 | 0.12 | 0.5 | 0.12 | 0.12 | 1 | 0.25 | 0.12 | 0.25 | 16 | 0.5 | 2 |
| **14b** | 0.5 | 2 | 0.12 | 0.25 | 1 | 0.25 | 0.25 | 4 | 0.5 | 1 | 0.5 | >32 | 1 | 8 |
| **14c** | 0.06 | 0.5 | ≤0.03 | 0.12 | 0.5 | ≤0.03 | 0.06 | 0.5 | 0.25 | ≤0.03 | 0.12 | 8 | 0.25 | 1 |
| **14d** | 0.25 | 2 | 0.12 | 0.12 | 1 | 0.12 | 0.25 | 8 | 0.5 | 0.12 | 0.25 | 16 | 0.5 | 1 |
| **16** | 4 | >32 | 16 | >32 | >32 | 8 | >32 | >32 | >32 | >32 | >32 | >32 | >32 | 16 |
| **19a** | ≤0.03 | 0.25 | ≤0.06 | 0.06 | 0.12 | ≤0.03 | ≤0.03 | 0.12 | 0.06 | ≤0.03 | 0.06 | 2 | 0.25 | 0.25 |
| **19b** | ≤0.03 | 0.5 | 0.06 | 0.06 | 0.12 | ≤0.03 | 0.06 | 1 | 0.06 | ≤0.03 | 0.06 | 4 | 0.25 | 0.25 |
| **19c** | ≤0.03 | 0.25 | ≤0.03 | ≤0.03 | 0.06 | ≤0.03 | ≤0.03 | 0.5 | 0.12 | ≤0.03 | 0.06 | 2 | 0.25 | 0.5 |

Metronidazole, a 5-nitroimidazole, is one of the most important drugs for the treatment of anaerobic bacterial infections. The activity of metronidazole against this panel was inconsistent with virtually no activity against *Lactobacillus acidophilus* (La), *Peptostreptococcus micros* (Pm), *Propionibacterium acnes* (Pas) and *Veillonella parvula* (Vp). Pretomanid, a 4-nitroimidazole, exhibited a similar spectrum of activity against this panel as metronidazole but with higher MICs. Linezolid was active against all the strains with MICs ranging from 0.06 to 2 μg/mL against the 13 anaerobic bacteria. Interestingly, the 1:1 combination of linezolid and pretomanid was about one dilution less active than linezolid, virtually reflecting the activity of linezolid in the mixture. Pretomanid did not appear to make any contribution to the activity of the combination.

Compound **5**, a conjugate molecule between linezolid and metronidazole, a 5-nitroimidazole, appeared to be more potent than either of its precursor antibiotics against all strains tested with MICs ranging from 0.06 to 1 μg/mL. The apparent synergistic effect of the 4-nitroimidazole-oxazolidione conjugate was more profound. The majority of the 12 compounds were significantly more potent than the combination of linezolid and pretomanid with the exception of compound **16**. Several compounds (**8a**, **8b**, **11a**, **14c**, **19a**, **19b** and **19c**) were 10-100 folds more potent against the majority of the tested strains than the combination.

The underlying mechanism for the apparent synergistic effect of this conjugate series is still unclear. A number of hypotheses are currently under consideration. The first hypothesis is that conjugation of a nitroimidazole to the oxazolidinone simply makes the oxazolidinone more potent. The nitroimidazole group plays a role of a substituent that make the oxazolidinone bind to the ribosomal RNA better. This hypnosis appears less plausible as the synergistic effect was only observed with anaerobic bacteria. We did not observe parallel improvement of potency against aerobic bacteria *Enterococcus faecium* and *Staphylococcus aureus* (Table 2). The second hypothesis is that the conjugate molecules bring additional nitromidazole inside of the cells by better penetration or avoidance of efflux. A third hypothesis is that nitroimidazole moiety acts synergistically when conjugated to an oxazolidinone pharmacophore. The high binding affinity of the oxazolidinone moiety to ribosomes or ribosomal components could bring the reactive species generated from the nitroimidazole moiety to the close affinity of the transcription/translation machinery and make them work more efficiently. This includes the possibility for irreversible covalent linking to ribosome or other enzymes associated with intrinsic oxazolidinone resistance. The last two hypotheses are supported by the mechanism of action study which indicated that both the nitroimidazole and the oxazolidinone functions contribute the antibacterial activity inside a *M. tuberculosis* cell (Table 1).

3. Experimental

3.1. Chemistry

**General**: Reference compounds linezolid, levofloxacin and metronidazole were purchased from ChemPacific or Sigma-Aldrich. Pretomanid (PA-824) and compound **5** were prepared according to published procedures [7]. All other compounds were synthesized by TenNor Therapeutics.

All starting materials are either purchased from commercial sources or prepared according to published procedures. Operations involving moisture and/or oxygen sensitive materials are conducted under an atmosphere of nitrogen. Flash chromatography is performed using silica gel 60 as normal phase adsorbent or C18 silica gel as reverse phase adsorbent. Nuclear magnetic resonance (NMR) spectra are recorded on a Varian 400 MHz magnetic resonance spectrometer. 1H NMR chemical shift are given in parts per million (*δ*) downfield from TMS. 1H NMR information is tabulated in the following format: number of protons, multiplicity (*s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet; *td*, triplet of doublet; *dt*, doublet of triplet), coupling constant(s) (*J*) in hertz. The prefix app is occasionally applied in cases where the true signal multiplicity is unresolved, and prefix *br* indicates a broad signal. High performance liquid chromatography (HPLC) analysis for the final compound is performed on Agilent 1100 instrument using a Waters Xterra RP18 column (5 μm, 4.6 mm × 250 mm) and gradient elution (solvent A, 20 mM NaH2PO4/acetonitrile, 60:40 v/v; solvent B, acetonitrile). HPLC purities for the final compound are ≥95%.

**(*R*)-3-(3-fluoro-4-(6-(((*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-yl)oxy)pyridin-3-yl)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (8a)** [11]. To a solution of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine (1.01g, 2.96 mmol) and (*R*)-3-(3-fluoro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-5-(hydroxymethyl) -oxazolidin-2-onein DMF (30 mL) was added a solution of K2CO3 (0.90 g, 6.51 mmol) in water (1 mL) and the mixture was purged with nitrogen. Pd(PPh3)4 (0.17 g, 0.15 mmol) was then added and the mixture was warmed with stirring under nitrogen at 80℃ for 3 hours. The solvents were completely removed under reduced pressure and the residue was partitioned between EtOAc and water. The extract was worked up and chromatographed on silica. Elution with EtOAc gave fore fractions, then elution with (1:20) MeOH/DCM gave **8a** as a yellow powder (460 mg, 33%). This general procedure of Suzuki coupling reaction was also applied for the synthesis of **8b**, **11b**, **14a-d**, and **19a-c**. 1H NMR (400 MHz, DMSO) *δ* 8.38 (s, 1H), 8.06(s, 1 H), 7.94-7.91 (m, 1 H), 7.63 (dt, *J* = 13.6, 2.4 Hz, 1 H), 7.57 (d, *J* = 8.8 Hz, 1 H), 7.46-7.43 (m, 1 H), 6.97 (d, *J* = 9.2 Hz, 1H), 5.76 (s, 1H), 5.24 (t, *J* = 5.6 Hz, 1H), 4.74-4.67 (m,2 H), 4.48-4.38 (m, 2 H), 4.13-4.09 (m, 1 H), 3.86 (dd, *J* = 8.8, 6.0 Hz, 1 H), 3.70-3.64 (m, 1H), 3.56-3.52 (m, 1 H), 3.14 (d, *J* = 5.2 Hz, 1 H). LC-MS (ESI): m/z = 472 (M+H)+.

**(*R*)-3-(3-fluoro-4-(2-(((*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-yl)oxy)pyrimidin-5-yl)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (8b)** [11]**.** The title compound was prepared by following the same procedure as described in preparation of **8a** except (*S*)-6-((5-bromopyrimidin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. (580 mg, 41%). 1H NMR (400 MHz, DMSO) δ 8.86 (s, 2 H), 8.06 (s, 1 H), 7.68 (d, *J* = 8.8 Hz, 1 H), 7.66 (t, *J* = 2.8 Hz, 1 H), 7.47 (dd, *J* = 8.8, 2.4 Hz, 1H), 5.74 (s, 1 H), 5.23 (t, *J* = 5.6 Hz,1 H), 4.77-4.70 (m, 2 H), 4.47 (s, 1 H), 4.1 1 (t, *J* = 9.0 Hz, 1 H), 3.86 (dd, *J* = 8.8, 6.0 Hz, 1H), 3.71-3.65 (m, 1 H), 3.57-3.53 (m, 1 H), 3.38-3.35 (m, 1 H). LC-MS (ESI): m/z = 473 (M+H)+.

***N*-(((*S*)-3-(2-fluoro-4'-((((*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-yl)oxy)methyl)-[1,1'-biphenyl]-4-yl)-2-oxooxazolidin-5-yl)methyl)acetamide (11a)** [11]**.** To a solution of (*S*)-6-((4-bromobenzyl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine (70.4 mg, 0.21 mmol) and (*S*)-*N*-((3-(3-fluoro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)-2-oxooxazolidin-5-yl) methyl)acetamide (86.0 mg, 0.227 mmol) in DMF (8 mL) was added a solution of Na2CO3 57.0 mg, 0.538 mmol) in water (1 mL) and the mixture was purged with nitrogen. Pd(PPh3)4 (24.0 mg, 0.021 mmol) was then added and the mixture was warmed with stirring under nitrogen at 85oC for 90 min. The solvents were completely removed under reduced pressureand the residue was partitioned between EtOAc and water. The extract was worked up and chromatographed on silica. Elution with EtOAc gave fore fractions, then elution with (1:9) MeOH/EtOAc gave **11a** as an off-white powder. (59.4 mg, 44%). 1H NMR (DMSO-d6, 400 MHz) δ 8.22 (t, *J* =5.8 Hz, 1H), 8.07 (s, 1H), 7.60-7.50 (m, 4H), 7.39 (dd, *J* =8.6, 2.2 Hz, 1H), 7.15 (d, *J* =8.8 Hz, 2H), 5.29 (br s, 1H), 4.79-4.70 (m, 2H), 4.70-4.64 (m, 2H), 4.42 (dd, *J* =13.8, 3.2 Hz, 1H), 4.35 (br d, *J* =13.8 Hz, 1H), 4.16 (t, *J* =9.0 Hz, 1H), 40.03 (m, 1H), 3.78 (dd, *J* =9.2, 6.5 Hz, 1H), 3.43 (t, *J* =5.5 Hz, 2H), 1.84 (s, 3H). LC-MS (ESI): m/z = 526 (M+H)+.

**(*R*)-3-(3-fluoro-4-(6-((((*S*)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-6-yl)oxy)methyl)pyridin-3-yl)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (11b)** [11]. The title compound was prepared by following the same procedure as described in preparation of **8a** except (*S*)-6-((5-bromopyridin-2-yl)methoxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. (360 mg, 38%). 1H NMR (400 MHz, DMSO) *δ* 8.68 (s, 1 H), 8.04 (s, 1 H), 7.96 (dt, *J* = 8.8, 1.6 Hz, 1 H), 7.65 (dd, *J* = 13.6, 2.4 Hz, 1 H), 7.60 (d, *J* = 8.8 Hz, IH), 7.46 (dd, *J* = 8.4, 2.8 Hz, 2 H), 5.74 (s, 1 H), 5.24 (t, *J* = 5.6 Hz, 1 H), 4.77-4.69 (m, 3 H), 4.48 (d, *J* = 12.0 Hz, 1 H), 4.35-4.22 (m, 3 H), 4.12 (t, *J* = 9.2 Hz, 1 H), 3.86 (dd, *J* = 8.8, 5.6 Hz, 1 H), 3.70-3.65 (m, 1 H), 3.58-3.52 (m, 1 H), 3.16-3.12 (m, 1 H). LC-MS (ESI): m/z = 486 (M+H)+.

**(5*R*)-3-(2-fluoro-4'-((2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazol-2-yl)methoxy)-[1,1'-biphenyl]-4-yl)-5-(hydroxymethyl)oxazolidin-2-one (14a)**. The title compound was prepared by following the same procedure as described in preparation of **8a** except (*S*)-6-((5-bromopyridin-2-yl)methoxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. 1H NMR (400 MHz, DMSO) δ 8.14 (s, 1H), 7.67 – 7.26 (m, 5H), 6.96 (d, *J* = 8.8 Hz, 2H), 5.20 (t, *J* = 5.6 Hz, 1H), 4.69 (td, *J* = 9.3, 3.6 Hz, 1H), 4.39 – 4.28 (m, 3H), 4.17 (d, *J* = 11.0 Hz, 1H), 4.07 (t, *J* = 9.0 Hz, 1H), 3.82 (dd, *J* = 8.8, 6.3 Hz, 1H), 3.65 (ddd, *J* = 12.2, 5.4, 3.2 Hz, 1H), 3.58 – 3.48 (m, 1H), 1.66 (s, 3H). LC-MS (ESI): m/z = 485 (M+H)+.

**(5*R*)-3-(2'-chloro-2-fluoro-4'-((2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazol-2-yl)methoxy)-[1,1'-biphenyl]-4-yl)-5-(hydroxymethyl)oxazolidin-2-one (14b)**. The title compound was prepared by following the same procedure as described in preparation of **8a** except 2-((4-bromo-3-chlorophenoxy)methyl)-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. 1H NMR (400 MHz, DMSO) δ 8.13 (s, 1H), 7.55 (dd, *J* = 12.5, 2.0 Hz, 1H), 7.37 (dd, *J* = 8.6, 2.0 Hz, 1H), 7.28 (dd, *J* = 15.5, 8.5 Hz, 2H), 7.13 (d, *J* = 2.5 Hz, 1H), 6.93 (dd, *J* = 8.6, 2.5 Hz, 1H), 5.20 (t, *J* = 5.6 Hz, 1H), 4.69 (dd, *J* = 9.1, 5.6 Hz, 1H), 4.41 – 4.29 (m, 3H), 4.16 (d, *J* = 11.0 Hz, 1H), 4.08 (t, *J* = 9.0 Hz, 1H), 3.82 (dd, *J* = 8.9, 6.1 Hz, 1H), 3.69 – 3.60 (m, 1H), 3.58 – 3.49 (m, 1H), 1.65 (s, 3H). LC-MS (ESI): m/z = 567 (M+H)+.

**(5*R*)-3-(3-fluoro-4-(6-((2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazol-2-yl)methoxy)pyridin-3-yl)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (14c)**. The title compound was prepared by following the same procedure as described in preparation of **8a** except 2-(((5-bromopyridin-2-yl)oxy)methyl)-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. 1H NMR (400 MHz, DMSO ) δ 8.34 (s, 1H), 8.15 (s, 1H), 7.94 – 7.87 (m, 1H), 7.68 – 7.55 (m, 2H), 7.45 (dd, *J* = 8.6, 2.1 Hz, 1H), 6.86 (d, *J* = 8.6 Hz, 1H), 5.22 (t, *J* = 5.6 Hz, 1H), 4.74 (td, *J* = 5.8, 2.7 Hz, 1H), 4.63 (s, 2H), 4.41 (d, *J* = 11.0 Hz, 1H), 4.25 – 4.09 (m, 2H), 3.88 (dd, *J* = 8.9, 6.2 Hz, 1H), 3.70 (ddd, *J* = 12.3, 5.5, 3.4 Hz, 1H), 3.58 (ddd, *J* = 12.3, 5.7, 4.1 Hz, 1H), 1.71 (s, 3H). LC-MS (ESI): M/Z = 486 (M+H)+.

**(5*R*)-3-(3-fluoro-4-(5-((2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazol-2-yl)methoxy)pyridin-2-yl)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (14d)**. The title compound was prepared by following the same procedure as described in preparation of **8a** except 2-(((6-bromopyridin-3-yl)oxy)methyl)-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. 1H NMR (400 MHz, DMSO) δ 8.32 (d, *J* = 2.9 Hz, 1H), 8.16 (d, *J* = 17.1 Hz, 1H), 7.90 (t, *J* = 9.0 Hz, 1H), 7.69 (d, *J* = 7.6 Hz, 1H), 7.58 (dd, *J* = 14.2, 1.9 Hz, 1H), 7.48 – 7.38 (m, 2H), 5.21 (t, *J* = 5.6 Hz, 1H), 4.70 (dd, *J* = 9.0, 5.7 Hz, 1H), 4.40 (dt, *J* = 11.0, 9.4 Hz, 3H), 4.17 (d, *J* = 11.0 Hz, 1H), 4.08 (t, *J* = 9.0 Hz, 1H), 3.83 (dd, *J* = 8.7, 6.3 Hz, 1H), 3.65 (ddd, *J* = 12.2, 5.2, 3.4 Hz, 1H), 3.59 – 3.49 (m, 1H), 1.67 (s, 3H). LC-MS (ESI): M/Z = 486 (M+H)+.

**(5*R*)-3-(3-fluoro-4-((5-((2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazol-2-yl)methoxy)pyridin-2-yl)oxy)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (16)**.

2-(((5-bromopyridin-2-yl)oxy)methyl)-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-b]oxazole (532 mg, 1.5 mmol), (R)-3-(3-fluoro-4-hydroxyphenyl)-5-(hydroxymethyl)oxazolidin-2-one (376 mg, 1.65 mmol ), Potassium carbonate(622mg, 4.5mmol), Cuprous iodide(286 mg, 1.5 mmol) and N,N,N',N'-Tetramethylethylenediamine (TMEDA) (174 mg, 1.5 mmol) were added in N,N-dimethylformamide (5mL). The mixture was stirred at 90℃ for 12h. The mixture was cooled to room temperature, then poured into water (50mL), the solvent was filtered and the filtered solid was pump dried. The crude product was further purified by silica chromatography column (DCM:MeOH = 100:2 ) to give (5*R*)-3-(3-fluoro-4-((5-((2-methyl-6-nitro-2,3-dihydroimidazo -[2,1-b]oxazol-2-yl)methoxy)pyridin-2-yl)oxy)phenyl)-5-(hydroxymethyl)oxazolidin-2-one(200mg,yield: 26.6%). 1H NMR (500 MHz, DMSO) δ 8.29 (s, 1H), 8.05 (s, 1H), 7.88 (d, *J* = 8.4 Hz, 1H), 7.64 – 7.50 (m, 2H), 7.41 (d, *J* = 8.5 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 4.70 (s, 1H), 4.48 (s, 2H), 4.23 – 4.01 (m, 3H), 3.83 (t, *J* = 7.3 Hz, 1H), 3.65 (d, *J* = 12.4 Hz, 1H), 3.53 (d, *J* = 12.2 Hz, 1H), 2.40 – 2.29 (m, 1H), 2.22 – 2.09 (m, 1H), 1.46 (s, 3H). LC-MS (ESI): m/z = 502 (M+H)+.

**(5*R*)-3-(3-fluoro-4-(6-((2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-7-yl)methoxy)pyridin-3-yl)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (19a)**. The title compound was prepared by following the same procedure as described in preparation of **8a** except 7-(((5-bromopyridin-2-yl)oxy)methyl)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. 1H NMR (400 MHz, DMSO) δ 8.32 (s, 1H), 8.06 (s, 1H), 7.91 (d, *J* = 8.2 Hz, 1H), 7.58 (dd, *J* = 22.0, 11.8 Hz, 2H), 7.42 (d, *J* = 8.0 Hz, 1H), 6.97 (d, *J* = 8.5 Hz, 1H), 5.21 (t, *J* = 5.4 Hz, 1H), 4.91 (s, 1H), 4.71 (d, *J* = 2.9 Hz, 1H), 4.57 (dt, *J* = 12.1, 7.7 Hz, 2H), 4.21 – 4.01 (m, 3H), 3.89 – 3.80 (m, 1H), 3.66 (d, *J* = 11.5 Hz, 1H), 3.60 – 3.49 (m, 1H), 2.29 (d, *J* = 13.4 Hz, 1H), 2.18 (dd, *J* = 15.1, 9.6 Hz, 1H). LC-MS (ESI): m/z = 486 (M+H)+.

**(5*R*)-3-(3-fluoro-4-(2-((2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-7-yl)methoxy)pyrimidin-5-yl)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (19b)**. The title compound was prepared by following the same procedure as described in preparation of **8a** except 7-(((5-bromopyrimidin-2-yl)oxy)methyl)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. 1H NMR (400 MHz, DMSO) δ 8.81 (d, *J* = 0.9 Hz, 2H), 8.07 (s, 1H), 7.70 – 7.62 (m, 2H), 7.48 – 7.41 (m, 1H), 5.22 (t, *J* = 5.6 Hz, 1H), 5.01 – 4.90 (m, 1H), 4.76 – 4.61 (m, 3H), 4.20 – 4.06 (m, 3H), 3.85 (dd, *J* = 8.8, 6.3 Hz, 1H), 3.66 (ddd, *J* = 12.3, 5.2, 3.4 Hz, 1H), 3.54 (ddd, *J* = 12.4, 5.6, 4.1 Hz, 1H), 2.30 (dd, *J* = 11.9, 2.4 Hz, 1H), 2.24 – 2.10 (m, 1H). LC-MS (ESI): m/z = 487 (M+H)+.

**(5*R*)-3-(3-fluoro-4-(6-((7-methyl-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazin-7-yl)methoxy)pyridin-3-yl)phenyl)-5-(hydroxymethyl)oxazolidin-2-one (19c)**. The title compound was prepared by following the same procedure as described in preparation of **8a** except 7-(((5-bromopyridin-2-yl)oxy)methyl)-7-methyl-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine was used instead of (*S*)-6-((5-bromopyridin-2-yl)oxy)-2-nitro-6,7-dihydro-5*H*-imidazo[2,1-*b*][1,3]oxazine. 1H NMR (500 MHz, DMSO) δ 8.29 (s, 1H), 8.05 (s, 1H), 7.88 (d, *J* = 8.4 Hz, 1H), 7.64 – 7.50 (m, 2H), 7.41 (d, *J* = 8.5 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 4.70 (s, 1H), 4.48 (s, 2H), 4.11 (dt, *J* = 17.0, 7.2 Hz, 3H), 3.83 (t, *J* = 7.3 Hz, 1H), 3.65 (d, *J* = 12.4 Hz, 1H), 3.53 (d, *J* = 12.2 Hz, 1H), 2.40 – 2.29 (m, 1H), 2.22 – 2.09 (m, 1H), 1.46 (s, 3H). LC-MS (ESI): m/z = 500 (M +H)+.

3.2. Biology

**Bacterial Strains**: Bacterial strains used in the current study are listed in Table 4. Strains in the *Mycobacterium tuberculosis* isogenic resistant mutant panel used for the mechanism of action study were generated and tested at Beijing Tuberculosis and Thoracic Tumor Research Institute (BTTTRI, Bejing, China). Strains of the spectrum panel were all ATCC stains and the testing was conducted at HD Biosciences (Shanghai, China). Strains of the anaerobic bacterial panel were ATCC strains and the test was conducted at Micomyx Inc. (Kalamazoe, Michigan, USA).

**Table 4.** List of bacterial strains used in the current study.

|  |  |  |  |
| --- | --- | --- | --- |
| Panel | Organism | ATCC No. | Testing Facility |
| Spectrum Panel | *Enterococcus faecalis* (Ef) | 700221 | HD Biosciences |
| *Staphylococcus aureus* (Sa) | 292213 |
| *Klebciella pneumonia* (Kp) | 43816 |
| *Acinetobacter baumannii* (Ab) | 19606 |
| *Pseudomonas aeruginosa* (Pa) | 27853 |
| *Escherichia coli* (Ec) | 25922 |
| *Clostrium difficile* (Cd) | 43255 |
| *M. tuberculosis* Isogenic Resistant Mutant Panel | *M. tuberculosis* H37Rv (Wild-Type) | 27294 | BTTTRI |
| *M. tuberculosis* P1 (Pretomanid-R) | Isogenic |
| *M. tuberculosis* P3 (Pretomanis-R) | Isogenic |
| *M. tuberculosis* L1 (Linezolid-R) | Isogenic |
| *M. tuberculosis* L3 (Linezolid-R) | Isogenic |
| Anaerobic Bacterial Panel | *Clostridium difficile* (Cd) | 700057 | Micromyx |
| *Lactobacillus acidophilus* (Lc) | 4356 |
| *Eggerthella lenta* (El) | 43055 |
| *Gardnerella vaginalis* (Gv) | 14018 |
| *Peptostreptococcus micros* (Pm) | 23195 |
| *Porphyromonas asaccharolytica* (Pas) | M3553 |
| *Propionibacterium acnes* (Pac) | 11892 |
| *Bacteroides fragilis* (Bf) | 25285 |
| *Bifidobacterium longum* (Bl) | 15707 |
| *Fusobacterium nucleatum* (Fn) | 10953 |
| *Mobiluncus mulieris* (Mm) | 35243 |
| *Prevotella bivia* (Pb) | 29303 |
| *Veillonella parvula* (Vp) | 17745 |
| *Helicobacter pylori* (Hp) | 43504 |

**Generation of Isogenic Mutant Panel:** The methods to induce and characterize the Mtb isogenic mutant strains resistant to pretomanid or linezolid have been described previously [12-13]. Briefly, 200 µl of 104 - 105 CFU/ml of *M. tuberculosis* H37Rv (ATCC27294) in 7H9 liquid culture medium at logarithmic growth phase were evenly spread on 7H11 solid medium containing 1 MIC (0.07 µg / ml) of pretomanid and blank control medium. After sealing, the plates were incubated at 37 °C under 5% CO2 for 3 to 4 weeks. Single colonies with good growth were collected from the drug-containing solid medium, milled and diluted to a concentration of 104-105 CFU/ml, and then inoculated onto 7H11 solid medium with pretomanid at 2 MIC (0.15 μg / ml) and blank control medium. The plates were then incubated under the same condition for another 3 to 4 weeks to observe the growth of single colonies. A single colony with robust growth was collected from the drug-containing medium and further subcultured on drug-containing solid medium with a 2-fold increase of concentration of pretomanid until the selection of a single colonies that grew well on solid medium with pretomanid concentration of 16 MIC. The phenotypic confirmation of the pretomanid resistant mutant strains were performed by Alamar Blue double dilution method [14]. The genotypic change of the resistant mutant strains were confirmed to be the T265C point mutation（TAC→CAC, Y89H）in the *ddn* gene. Similar sequential drug selection method was applied to obtain linezolid resistant mutant trains. The genotypic change of the linezolid resistant mutant strains was found to be the point mutation of the *rplC* (T460C).

**Media:** Media were prepared according to guidelines from CLSI. The test medium used for the anaerobic bacterial was supplemented Brucella broth (BD, Lot No. 6278735) containing 5 μg/mL hemin (Sigma, Lot No. SLBC4685V), 1 mg/mL Vitamin K1 (Sigma, Lot No. MKBN5958V), and 5% (v/v) laked horse blood (LHB, Cleveland Scientific, Lot No. 385663). Brucella broth (BD) supplemented with 10% fetal bovine serum (FBS, Gibco, Lot No. 1709261) was used for testing of *H. pylori*. Cation-adjusted Mueller Hinton Broth (CAMHB) media was used for the spectrum determination against the spectrum panel. Difco Middlebrook 7H9 Broth (Catalog No. 271310) supplemented with 0.2% (v/v) glycerol, 0.05% Tween 80, and 10% (v/v) albumin-dextrosecatalase (BBL Middlebrook ADC Enrichment, Catalog No. 212352) (7H9-ADC-T) was used for the MIC assay against the *M. tuberculosis* isogenic resistant mutant panel.

**Minimum Inhibitory Concentration Testing:** The MIC test against the spectrum panel was conducted at HB Biosciences, following the broth microdilution method per CLSI guidance. The six ESKAPE ATCC strains were recovered in Trypticase Soy Agar (TSA) plates and tested with drug in CAMHB. Anaerobic bacterial *C. difficile* strains were recovered on TSA agar, grown and tested with compounds in Blucella broth with 5 µg/ml chlorhematin and 10 µg/ml vitamin K1 in an anaerobic chamber.

The MIC assay against the Mtb isogenic resistant mutant strains was performed at BTTTRI by the microplate Alamar blue assay [14]. Pretomanid, linezolid and metronidazole were used as comparators. The H37Rv strain and its derived isogenic resistant mutant strains were grown for 1-2 weeks at 370C and adjusted to a turbidity of McFarland 1 at 107CFU/mL and diluted 1:20. Twofold dilutions of testing compounds and comparators were prepared in 7H9-ADC-TG in a volume of 100 µl in 96-well, black, clear-bottom microplates (BD Biosciences, Franklin Lakes, NJ). Bacterial cells (100 µl containing 2 x 105 CFU) was added, yielding a final testing volume of 200 µl. The plates were incubated at 37°C, on day 7, 12.5 µl of 20% Tween 80 and 20 µl of Alamar blue were added to all wells. After incubation at 37°C for 16 to 24 h, the fluorescence was read at an excitation of 530 nm and an emission of 590 nm. The MIC was defined as the lowest concentration effecting a reduction in fluorescence of ≥ 90% relative to the mean of replicate bacterium-only controls.

The MIC assay against anaerobic bacterial strains was performed at Micromyx by following the procedure described by CLSI [15-17]. A standardized inoculum of each organism was prepared per CLSI methods. Colonies were picked from the primary plate and a suspension was prepared to equal to a 0.5 McFarland turbidity standard. Anaerobic suspensions were diluted 1:10 in Brucella broth with 5% laked horse blood and each well was inoculated with 10 μL using a multichannel pipette in the Bactron anaerobe chamber, resulting in a final cell density of approximately 5105 CFU/mL (5104 CFU/mL for Clostridium spp.). For the *H. pylori* strain, colonies were picked from the primary plate and a suspension was prepared to equal a 2.0 McFarland turbidity standard. Suspensions were diluted 1:15 in Brucella broth with 10% FBS, then transferred to compartments of sterile reservoirs divided by length (Beckman Coulter). The Biomek 2000 was used to inoculate the plates. Daughter plates were placed on the Biomek 2000 work surface in reverse orientation so that plates were inoculated from low to high drug concentration. The Biomek 2000 delivered 10 μL of standardized inoculum into each well of the appropriate daughter plate for an additional 1:20 dilution. Anaerobe plates were placed in an anaerobic box with GasPak sachets (BD), and were incubated anaerobically for 46-48 hr at 35-37°C. *H. pylori* was incubated for 72 hr in a microaerophilic atmosphere using boxes with GasPak EZ Campy sachets (BD) prior to reading. After incubation, plates were viewed from the bottom using a plate viewer. An un-inoculated solubility control plate was observed for evidence of drug precipitation. MIC values were read and recorded as the lowest concentration of drug that inhibited visible growth of the organism.

**Cytotoxicity Assay:** The cytotoxicity assay followed the previously published protocol [14]. Briefly, Vero cells in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) were incubated in a humidified atmosphere of 5% CO2 at 37°C to reach confluent and then diluted to 4 x 105 cells/ml. Threefold serial dilutions of the stock solutions resulted in final concentrations of 64 to 0.26 µg/ml in a final volume of 100 µl. Cytotoxicity testing was performed in a transparent 96-well microplate. After incubation at 37°C for 48 h, the medium was removed, and the monolayers were washed twice with 100 µl of warm Hanks balanced salt solution (HBSS). Warm medium and freshly made methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) were added to each well, and then the plates were incubated for 4 h, after which the absorbance was determined at 492 nm.

4. Conclusions

A series of 4-nitroimidazole and oxazolidione conjugate molecules were designed, synthesized and evaluated based on previous structure-activity relationship information. The dual mechanism of action of this series was demonstrated against *Mycobacterium tuberculosis* by utilizing an isogenic mutant panel resistant to either pretomanid or linezolid. Compounds in the series are highly active against a panel of clinically important anaerobic bacteria. Strong synergy was observed as compared to the combination of linezolid and pretomanid. The nitroimidazole-oxazolidinone conjugate molecules hold potential for the treatment of anaerobic bacterial infections.

**Author Contributions:** Zhenkun Ma, Charles Z. Ding, Anthony Simon Lynch, Anna M. Upton, Christopher B. Cooper and William A. Denny conceived the compound series and were responsible for the initial design, synthesis and evaluation of the conjugate series. Zhijun Zhuang, Dawei Wan, Jun Ding and Qian Zhang conducted the synthesis and characterization of the conjugate molecules of the current study. Shijie He, Xiaomei Wang and Ying Yuan coordinated and oversaw compound testing. Yu Lu generated and tested against the Mtb isogenic resistant mutant panel.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

References

1. Hashemian, S. M. R.; Farhadi, T.; Ganjparvar, M., Linezolid: a review of its properties, function, and use in critical care. *Drug Des. Devel. Ther.* **2018**, *12*, 1759-1767.
2. Cellitti, Susan E.; Shaffer, J.; Jones, David H.; Mukherjee, T.; Gurumurthy, M.; Bursulaya, B.; Boshoff, Helena I.; Choi, I.; Nayyar, A.; Lee, Yong S.; Cherian, J.; Niyomrattanakit, P.; Dick, T.; Manjunatha, Ujjini H.; Barry, Clifton E., III; Spraggon, G.; Geierstanger, Bernhard H., Structure of Ddn, the Deazaflavin-Dependent Nitroreductase from *Mycobacterium tuberculosis* Involved in Bioreductive Activation of PA-824. *Structure* **2012**, *20*, 101-112.
3. Singh, R.; Manjunatha, U.; Boshoff, H. I. M.; Ha, Y. H.; Niyomrattanakit, P.; Ledwidge, R.; Dowd, C. S.; Lee, I. Y.; Kim, P.; Zhang, L.; Kang, S.; Keller, T. H.; Jiricek, J.; Barry, C. E., PA-824 Kills Nonreplicating *Mycobacterium tuberculosis* by Intracellular NO Release. *Science* **2008**, *322*, 1392-1395.
4. Conradie, F.; Diacon, A. H.; Ngubane, N.; Howell, P.; Everitt, D.; Crook, A. M.; Mendel, C. M.; Egizi, E.; Moreira, J.; Timm, J.; McHugh, T. D.; Wills, G. H.; Bateson, A.; Hunt, R.; Van Niekerk, C.; Li, M.; Olugbosi, M.; Spigelman, M., Treatment of Highly Drug-Resistant Pulmonary Tuberculosis. *N. Engl. J. Med.* **2020**, *382*, 893-902.
5. Ma, Z.; Lynch, A. S., Development of a Dual-Acting Antibacterial Agent (TNP-2092) for the Treatment of Persistent Bacterial Infections. *J. Med. Chem.* **2016**, *59*, 6645-6657.
6. Ippolito, J. A.; Kanyo, Z. F.; Wang, D.; Franceschi, F. J.; Moore, P. B.; Steitz, T. A.; Duffy, E. M., Crystal Structure of the Oxazolidinone Antibiotic Linezolid Bound to the 50S Ribosomal Subunit. *J. Med. Chem.* **2008**, *51*, 3353-3356.
7. Varshney, V.; Mishra, N. N.; Shukla, P. K.; Sahu, D. P., Synthesis of nitroimidazole derived oxazolidinones as antibacterial agents. *Eur. J. Med. Chem.* **2010**, *45*, 661-666.
8. Markad, S. D.; Kaur, P.; Kishore Reddy, B. K.; Chinnapattu, M.; Raichurkar, A.; Nandishaiah, R.; Panda, M.; Iyer, P. S., Novel lead generation of an anti-tuberculosis agent active against non-replicating mycobacteria: exploring hybridization of pyrazinamide with multiple fragments. *Med. Chem. Res.* **2015**, *24*, 2986-2992.
9. Goto, F.; Takemura, N.; Otani, T.; Hasegawa, T.; Tsubouchi, H.; Utsumi, N.; Fujita, S.; Kuroda, H.; Shitsuta, T.; Sasaki, H. 1-substituted-4-nitroimidazole compound and process for producing the same. U.S. Patent 7,368,579, 2008.
10. Thompson, A. M.; Denny, W. A.; Blaser, A.; Ma, Z. Nitroimidazooxazine and nitroimidazooxazole analogues and their uses. U.S. Patent 8,293,734, 2012.
11. Ding, C. Z.; Lu, G.; Combrink, K.; Chen, D. D.; Song, M.; Wang, J.; Ma, Z.; Palmer, B. D.; Blaser, A.; Thompson, A. M. Bicyclic nitroimidazole-substituted phenyl oxazolidinones. U.S. Patent 7,666,864, 2010.
12. Hu, M.H.; Wang, B.; Fu, L.; Xu, J.; Lu, Y. Induction and stability of M*ycobacterium tuberculosis* resistance to PA-824 in vitro. *J. Chinese Ant.* **2011**, *42*, 144-148.
13. Hu, M.H.; Wang, B.; Fu, L.; Xu, J.; Lu, Y. Induction in vitro and stability of *Mycobacterium tuberculosis* resistance to Linezolid. *Chin. J. Antibio.* **2017**, *39*, 400-405.
14. Lu, Y.; Zheng, M.; Wang, B.; Fu, L.; Zhao, W.; Li, P.; Xu, J.; Zhu, H.; Jin, H.; Yin, D.; Huang, H.; Upton, A. M.; Ma, Z., Clofazimine Analogs with Efficacy against Experimental Tuberculosis and Reduced Potential for Accumulation. *Antimicrob. Agents Chemother.* **2011**, *55*, 5185-5193.
15. Clinical and Laboratory Standards Institute (CLSI). Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline Second Edition. CLSI document M45-A2. Clinical and Laboratory Standards Institute: Wayne, Pennsylvania, USA, 2010.
16. Clinical Laboratory and Standards Institute (CLSI). Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard-Eighth Edition. CLSI document M11-A8. Clinical and Laboratory Standards Institute: Wayne, Pennsylvania, USA, 2012.
17. Clinical Laboratory and Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Seventh Informational Supplement. CLSI document M100-S27. Clinical and Laboratory Standards Institute: Wayne, Pennsylvania, USA, 2017.

**Sample Availability:** All samples of the compounds are available from the authors.

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