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Review Article

Antibacterial Activity of N-Substituted-Amino Acid Triazolyl Oxazolidinones against Clinical Isolates of Linezolid-Resistant Gram-Positive Cocci and MDR *Mycobacterium tuberculosis*

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Abstract

A small series of *N*-substituted-alaninyl and *N*-substituted-glycinyl-triazolyl oxazolidinone derivatives were evaluated against susceptible and drug-resistant Gram-positive cocci, including multidrug-resistant mycobacterial and linezolid-resistant clinical isolates.

The 5-nitrothiophene-2-carbonyl (PH-224(*D*) and PH-232(*L*)) and the 3,5-dinitrobenzoyl (PH-214(*D*)) alanine containing derivatives demonstrated the most potent ant mycobacterial activity with MIC values of 0.065, 0.26 µM and 1.1 µM, respectively, under aerobic conditions compared with rifampicin (MIC: 0.0067 µM). These three compounds also demonstrated MBC (minimum concentration values required to achieve a 2-log kill in 21 days) of 0.39 and 0.17 and 1.6 µM for PH-224(*D*), PH-232(*L*) and PH-214(*D*), respectively. PH-214(*D*), PH-224(*D*) and PH-232(*L*) also showed potent activity in low oxygen recovery assay (LORA) with MIC values, of 0.24 and 0.27, and 0.48 µM, respectively, indicating their potential usefulness in treating latent tuberculosis, which is often presumed untreatable. The 5-nitrothiophene-2-carbonyl substituted derivatives PH-224(*D*) and PH-232(*L*) were also mostly potent against fluoroquinolone-resistant (FQ-R1), isoniazid-resistant (INH-R1 and INH-R2) and rifampicin-resistant (RIF-R1 and RIF-R2) mycobacterial isolates. In addition, they showed significant intracellular antibacterial activity which is comparable to isoniazid. Against linezolid-resistant strains, the 5-nitro-2-furoyl alaninyl derivatives PH-223(*D*) and PH-223(*L*) and 5-nitrothiophene-2-carbonyl substituted alaninyl derivatives PH-224(*D*) and the 5-nitro-2-furoyl glycinyl derivatives PH-145 and PH-189 showed potent activity against four LNZ-resistant CNS-strains but were less active against the two LNZ-resistant *E. faecalis* strains.

INTRODUCTION

The emergence and spread of Gram-positive and Gram-negative bacterial resistance continue to pose urgent threat on global health, prompting the World Health Organization (WHO) to declare that the "post antibiotic" era is nearby. Currently, antimicrobial resistance (AMR) causes nearly 700,000 or more deaths annually and it has been estimated that this number could increase to about 10 million by 2050 [1,2]. The WHO classified AMR as an emerging threat with permanent humanitarian and economic consequences if not tackled aggressively [3]. In 2019, the WHO reported that about 10 million people fell ill with tuberculosis (TB) worldwide with a total of 1.4 million deaths (including 208,000 people with HIV). TB is classified as one of the top 10 causes of death and the leading cause from a single infectious agent (above HIV/AIDS). Furthermore, the WHO reported a 10% increase in multidrug-resistant and rifampicin-resistant TB (MDR/RR-TB) worldwide between 2018 and 2019, and thus rated MDR-TB as both a public health crisis and a health security threat [4]. There is therefore a pressing need for significant efforts to be expended on the discovery and development of newer and more effective antibacterial agents with lower potential for the development of resistance.

Oxazolidinones are synthetic potent antibacterial agents effective against multi-drug resistant Gram-positive bacteria, including staphylococci, streptococci, enterococci, and Mycobacterial strains. Linezolid (Lzd, Zyvox®, 1, Figure 1) is the first member of this class of antibacterial agents approved for clinical use and in 2014 Tedizolid (Sivextro®, 2, Figure 1) with

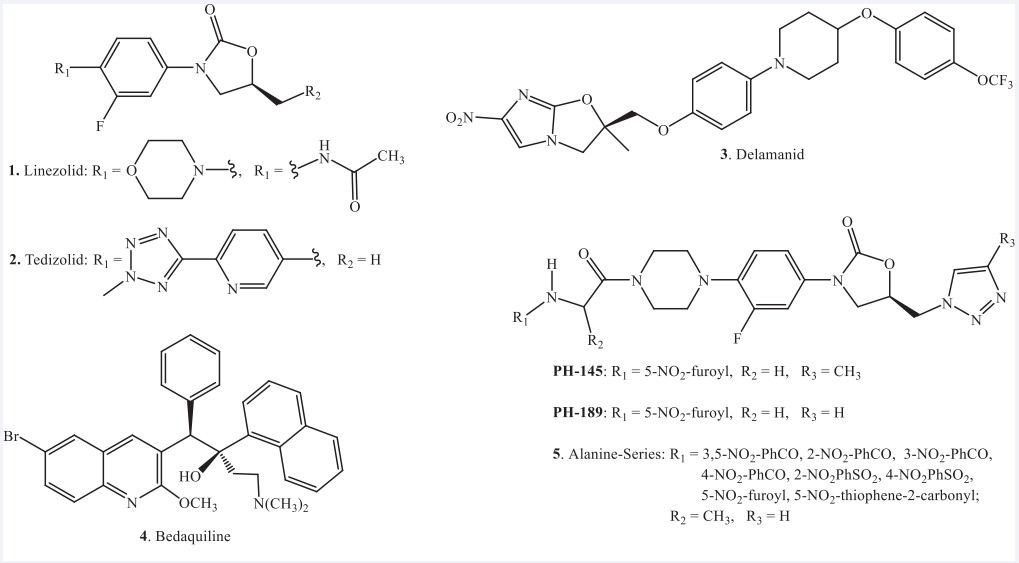


Figure 1 Structures of antibacterial agents.

comparable antibacterial activity [5-9]. Linezolid inhibits protein synthesis by binding to a site on the bacterial 23S ribosomal RNA of the 50S subunit. This prevents formation of the 70S ribosomal unit; therefore, inhibiting protein synthesis [10,11]. Both linezolid and tedizolid are clinically effective against drug-resistant Gram-positive bacterial strains, including vancomycin-resistant *Enterococcus faecium*, methicillin- and vancomycin-resistant *Staphylococcus aureus* and penicillin-resistant pneumococci. To further complicate the clinical management of Gram-positive bacterial infections, linezolid-resistant strains of *S. aureus* (LRSA), *S. haemolyticus* (LRSH) and enterococci (LRE) have been reported in the clinic [12-14]. Moreover, linezolid is also active against mycobacterial species, including *Mycobacterium tuberculosis*, with MIC values ranging from 0.125 to 1 µg/ml and MIC_{50} and MIC_{90} values of 0.5 µg/ml and 1 µg/ml, respectively [15-17]. As a result, Lzd has been used outside of the labeled indications to treat recalcitrant cases of MDR- and extensively drug-resistant TB (XDR-TB) [18]. In addition, some of the newer TB drugs, namely bedaquiline, a diarylquinoline (3, Figure 1), and delamanid, a nitroimidoxazole (4, Figure 1) have received conditional stringent regulatory approval for their uses in MDR-TB [19-22] adding to the anti-TB armamentarium. However, both drugs have serious adverse effects [23].

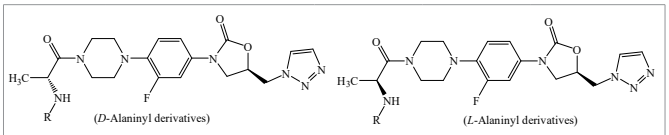
Our laboratory has been involved in the synthesis and antibacterial susceptibility testing of novel triazolymethyl oxazolidinones against resistant bacterial strains [24-27]. We have reported the potent antibacterial activity of the glycinyl 1*H*-1,2,3-triazolyl oxazolidinones (PH-145 and PH-189, Figure 1) against drug-resistant Gram-positive cocci and resistant *M. tuberculosis* [27-28]. Moreover, we have also reported on the antibacterial activity of the *N*-substituted *D*/*L*-alaninyl 1*H*-1,2,3-triazolyl oxazolidinones (5, Figure 1 and Table 1) against standard Gram-positive cocci and *Moraxella catarrhalis* strains [29]. In this study, we investigated the antibacterial activity of the nitro-containing *N*-substituted-glycinyl and - (*D*/L)-alaninyl triazolyl oxazolidinone derivatives against susceptible and resistant *M. tuberculosis*, *M. abscessus, M. avium* strains and linezolid-resistant Gram-positive clinical isolates.

RESULTS AND DISCUSSION

The compounds evaluated in this study were previously synthesized and reported from our laboratory [27,29]. The potent antibacterial activities of *N*-substituted-glycinyl oxazolidinone derivatives PH-145 and PH-189 (Figure 1) against susceptible and resistant clinical isolates of Gram-positive cocci and the Mycobacterial strain *Mtb* H37Rv have been previously reported [27,28]. Furthermore, we have also reported the moderate to strong antibacterial activities of the *N*-substituted-alaninyl oxazolidinone derivatives PH-214(*D*), PH-215(*D*), PH-217(*D*), PH-218(*D*), PH-219(*D*), PH-220(*D*), PH-224(*D*), PH-227(*L*), PH-228(*L*), PH-229(*L*), PH-230(*L*), PH-232(*L*), and PH-231(*L*) [Table 1] against standard strains of Gram-positive cocci [29].

Based on the potent antibacterial activities of these two series of *N*-substituted-amino acid oxazolidinone derivatives, we were prompted to evaluate the antibacterial activities of the 13 *N*-substituted-alaninyl derivatives against susceptible and resistant *M. tuberculosis* strains of *Mtb* H37Rv. The ant mycobacterial susceptibility testing using the *M. tuberculosis* (*Mtb*) H37Rv, *M. abscessus* subsp. *bollettii* 103 and *M. avium* subsp. *avium* 2285 (S) were performed by the National Institute of Health / National Institute of Allergy and Infectious Disease (NIH/NIAID, USA). The 50% (IC_{50}, µM) and 90% (IC_{90}, µM) inhibitory concentrations and the minimum inhibitory concentration (MIC, µM) data from the initial screening of these 13 derivatives are presented in [Table 1]. The compounds demonstrated moderate to strong anti-mycobacterial activity with MIC in the range of

Table 1: MIC's of oxazolidinones against *Mycobacterium tuberculosis* H37Rv (n=5)



Compound code	R_1	MIC (μM)	IC_{50} (μM)	IC_{90} (μM)
PH-214(D)	3,5-dinitrobenzoyl	1.6	0.68	1.7
PH-215(D)	4-nitrobenzenesulfonyl	22	7.6	23
PH-217(D)	2-nitrobenzenesulfonyl	19	6.4	20
PH-218(D)	4-nitrobenzoyl	4.9	2.1	5.3
PH-219(D)	2-nitrobenzoyl	9.7	2.6	11
PH-220(D)	3-nitrobenzoyl	4.9	2.4	5.6
PH-227(L)	3-nitrobenzoyl	3.8	1.5	4.2
PH-228(L)	4-nitrobenzoyl	4.8	1.7	5.6
PH-229(L)	4-nitrobenzoyl	9.6	2.3	10
PH-230(L)	4-nitrobenzenesulfonyl	19	5.7	26
PH-231(L)	2-nitrobenzenesulfonyl	18	6.4	20
PH-224(D)	5-nitrothiophene-2-carbonyl	0.39	0.11	0.41
PH-232(L)	5-nitrothiophene-2-carbonyl	0.69	0.19	0.65
Rifampicin		0.0067	0.0034	0.0057

0.39 to 22 μM compared with rifampicin (MIC: 0.0067 μM). From these 13 compounds, the seven most active compounds, namely, PH-214(*D*), PH-218(*D*), PH-220(*D*), PH-224(*D*), PH-227(*L*), PH-228(*L*), and PH-232(*L*) were selected for further evaluation under a variety of conditions in comparison to rifampicin. The antibacterial activity profiles, namely, the MIC and minimum bactericidal concentration (MBC, μM) values of these seven compounds against Mycobacterial strains of *Mtb* H37Rv, *M. abscessus* subsp. *bollettii* 103 and *M. avium* subsp. *avium* 2285 (S) under aerobic conditions are presented in [Table 2]. From this study, the 5-nitrothiophene-2-carbonyl containing derivatives PH-224(*D*) and PH-232(*L*) were the most active compounds against Mycobacterial strains of *Mtb* H37Rv with MIC values of 0.065 and 0.26 μM, respectively, compared with rifampicin (MIC: 0.0067 μM). While the MBC defined as the minimum concentration values required to achieve a 2-log kill in 21 days for PH-224(*D*) and PH-232(*L*) are 0.39 and 0.17 μM, respectively. Moreover, the 3,5-dinitrobenzoyl derivative PH-214(*D*) ranked third in activity next to these with MIC value of 1.1 μM and MBC of 1.6 μM. However, there is no clear-cut direct correlation between the antibacterial activity and the stereochemistry (*D*/*L*) of the alaninyl moiety, since the most active compound is PH-224(*D*) and the least active is PH-220(*D*).

Among the non-tuberculous mycobacterial (NTM) species, *Mycobacterium avium* complex (MAC), *Mycobacterium abscessus* and other mycobacterial species are responsible for most cases of pulmonary infections [30]. They can also cause systemic and / or disseminated infections. The activity (MIC, Table 2) of the compounds against *M. abscessus* subsp. *bollettii* 103 and *M. avium* subsp. *avium* 2285 (S) was assessed under aerobic conditions by measuring bacterial growth in the presence of the test compounds compared with Rifampicin. These oxazolidinones demonstrated MIC values in the ranges of 2.1 to 10, and 0.78 to 6.2 μM against *M. abscessus* subsp. *bollettii* 103 and *M. avium* subsp. *avium* 2285, respectively in comparison to rifampicin with MIC values of 3.0 and 0.10 μM. However, the 5-nitrothiophene-2-carbonyl derivatives PH224 (D) and PH232 (L) showed most potent activity against *M. avium* and *M. abscessus* with MIC values of 0.78 and 2.1 μM, respectively.

Mycobacterium tuberculosis (Mtb) is an obligate aerobe requiring oxygen for replication, however, *Mtb* strain can reside in a state of latency or Non-Replicating Persistent (NRP) state representing a hypoxic condition, which is often presumed untreatable. An approximate 1.7 billion people are latently infected with TB and on reactivation many of these infections are drug resistant [31]. The seven most active compounds were tested under low oxygen recovery assay (LORA) to assess their effectiveness against *Mtb* strain in NRP state. In this assay, bacterial growth was measured by luminescence, and rifampicin and metronidazole were included in the study as positive control for aerobic and anaerobic killing of *Mtb*, respectively. The LORA MIC values are presented in [Table 3]. Three compounds PH-214(*D*), PH-224(*D*) and PH-232(*L*) showed potent activity in LORA state with MIC values, of 0.24 and 0.27, and 0.48 μM, respectively, compared with rifampicin (MIC = 0.0016 μM) and metronidazole (MIC = 130 μM). Under LORA, PH-242(*D*) and PH-232(*L*) demonstrated almost comparable activity (IC_{50} and IC_{90} values) to rifampicin [Table 3]; while the other four tested derivatives PH-218(*D*), PH-220(*D*), PH-227(*D*) and PH-228(*L*) were significantly less active in LORA with MIC values in the range of 4.1 to 10 μM. Furthermore, out of these seven most active derivatives, the 5-nitrothiophene-2-carbonyl substituted derivatives PH-224(*D*) and PH-232(*L*) showed the most potent activity against fluoroquinolone-resistant (FQ-R1), isoniazid-resistant (INH-R1 and INH-R2) and rifampicin-resistant (RIF-R1 and RIF-R2) mycobacterial isolates. PH-224(*D*) demonstrated MIC values of 0.36, 0.40, 0.14, 0.042 and 0.52 μM against these strains, in comparison to PH-232(*L*) with MIC values of 1.4, 0.99, 0.38, 0.24 and 0.31 μM against similar strains [Table 4]. The rest of the 5-*N*-substituted-alaninyl derivatives demonstrated only modest activity with MIC values in the ranges of 7.8-29, 4.6-23, 2.2-14, 1.1-8.5 and 12-30 μM, against similar strains, respectively.

The antibacterial activity of the seven compounds against intracellular (macrophage) *Mtb* was performed using the THP-1 cells infected with *M. tuberculosis*, and the IC_{50} (μM) was determined as the concentration of compound causing 50% loss in viability [Table 5]. From this study, only the 5-nitrothiophene derivatives PH-224(*D*) and PH-232(*L*) showed any significant intracellular antibacterial activity with IC_{50} values of 0.50 and 1.2 μM in comparison to isoniazid with IC_{50} value of 0.14 μM. The cytotoxicity of the seven most active derivatives towards eukaryotic cells was determined and the IC_{50} (μM) are presented in [Table 5]. Of the compounds tested, PH-224(*D*) and PH-232(*L*) showed reduced levels of cytotoxicity with IC_{50} values of 12 and 16 μM, respectively, compared with straurosporine as control with IC_{50} value of 0.013 μM. The other derivatives demonstrated cytotoxicity at IC_{50} value of >50 μM. Straurosporine is a cell permeable alkaloid and an inhibitor of tumor cell growth capable

Table 2: MIC, MBC, IC50 and IC90 values of oxazolidinones under aerobic conditions against Mtb H37Rv (n=9), M. abscessus (n=1) and M. avium (n=1)

Compd code	M.tb H37Rv (n=9)				*M. abscessus*	*M. avium*
	MIC (µM)	MBC (µM)	IC_{50} (µM)	IC_{90} (µM)	MIC (µM)	MIC (µM)
PH214(D)	1.1	1.6	0.19	0.21	10	1.6
PH218(D)	3.3	25	1.4	2.3	8.0	6.2
PH220(D)	7.0	24.5	2.1	4.5	13	6.2
PH224(D)	0.065	0.39	0.20	0.24	6.3	0.78
PH227(L)	3.1	19	0.97	2.1	3.6	6.2
PH228(L)	4.4	4.8	0.78	2.1	4.1	6.2
PH232(L)	0.26	0.17	0.37	0.42	2.1	3.1
Rifampicin	0.0067	N/A	0.0038	0.0092	3.0	0.10

Table 3: MIC, IC_{50} and IC_{90} values under anaerobic conditions against M.tb H37Rv (n=9)

Compound code	Low oxygen MIC (µM)	Low oxygen IC_{50} (µM)	Low oxygen IC_{90} (µM)	Normal oxygen MIC (µM)	Normal oxygen IC_{50} (µM)	Normal oxygen IC_{90} (µM)
PH214(D)	0.24	0.19	0.21	0.42	0.10	0.20
PH218(D)	4.1	1.4	2.3	5.6	1.2	2.5
PH220(D)	10	2.1	4.5	5.8	1.6	3.0
PH224(D)	0.27	0.20	0.24	0.017	0.0051	0.0091
PH227(L)	4.7	0.97	2.1	3.5	0.67	1.5
PH228(L)	6.3	0.78	2.1	5.3	0.39	1.4
PH232(L)	0.48	0.37	0.42	0.22	0.052	0.1
Rifampicin	0.027	0.0016	0.0061	0.0079	0.0017	0.0036
Metronidazole	130	49	78	>200	>200	>200

Table 4: MIC, IC_{50} and IC_{90} (µM) values against resistant *M.tb* isolates under aerobic conditions

Compd Code	FQ-R1[a]			INH-R1[b]			INH-R2[c]			RIF-R1[d]			RIF-R2[e]		
	MIC	IC_{50}	IC_{90}	MIC	IC_{50}	IC_{90}	MIC	IC_{50}	IC_{90}	MIC	IC_{50}	IC_{90}	MIC	IC_{50}	IC_{90}
PH214(D)	7.8	1.5	17	4.6	1.6	5.3	2.2	0.23	2.4	1.1	0.11	1.3	12	1.5	15
PH218(D)	25	5.4	32	20	7	24	8.4	2.1	10	5.6	1.2	7.1	27	7.7	33
PH220(D)	29	8.7	36	23	8.9	26	14	2.8	17	8.5	1.7	11	30	12	36
PH224(D)	0.36	0.089	0.49	0.40	0.11	0.53	0.14	0.03	0.17	0.042	0.011	0.045	0.52	0.12	0.68
PH227(L)	16	4	19	10	4.1	12	8.2	1.3	11	4.1	0.64	5.5	33	5.5	42
PH228(L)	17	3.7	19	8.6	3.6	10	11	1.1	13	6.5	0.52	7.9	23	4.6	28
PH232(L)	1.4	0.28	1.8	0.99	0.30	1.20	0.38	0.088	0.46	0.24	0.065	0.31	1.7	0.32	2.4

[a]Fluoroquinolone-resistant strains. [b,c] Isoniazid-resistant strains. [d,e]Rifampicin-resistant strains

Table 5: Cytotoxicity and Intracellular activity

Compound code	Intracellular Activity		Cytotoxicity
	IC_{50} (µM)	IC_{90} (µM)	IC_{50} (µM)
PH214(D)	3.5	16	>50
PH218(D)	7.8	49	>50
PH220(D)	7.3	> 50	>50
PH224(D)	0.50	11	12
PH227(L)	4.2	> 50	>50
PH228(L)	5.4	46	>50
PH232(L)	1.2	11	16
Straurosporine*	N/A	N/A	0.013
Isoniazid*	0.14	0.22	N/A

* Control

of inducing cell death via intrinsic apoptotic pathways. This data showed that our compounds are relatively less toxic compared with straurosporine, the control compound.

Based on the potent activity of these compounds against resistant mycobacterial strains, we further evaluated the antibacterial activities of the *N*-substituted-alaninyl and *N*-substituted-glycinyl oxazolidinone derivatives against resistant clinical isolates of Gram-positive cocci including linezolid resistant strains, namely, *E. faecalis* (E-test resistant at 16µg and 48µg) and Methicillin-resistant coagulase negative Staphylococci (MR-CNS, E-test resistant at 32µg, 48µg and 256µg). The comparative antibacterial activities of the tested compounds against standard, susceptible and resistant bacterial strains are presented in Table 6. In general, all the *D*/*L*-alaninyl derivatives were active against all the standard reference and susceptible strains evaluated with MIC values in the range of 0.5 to 8µg/ml. However, the 5-nitro-2-furoyl (PH-223(*D*) and PH-223(*L*)) and the 5-nitrothiophene-2-carbonyl derivatives (PH-224(*D*) and PH-232(*L*)) showed the most potent activity with MIC values in the ranges of 0.25 to 2µg/ml and 0.5 to 8µg/ml, respectively. These were comparable in antibacterial activity to the previously evaluated glycinyl derivatives PH-145 and PH-189 with MIC values of <0.5µg/ml [29]. Moreover, the 5-nitro-2-furoyl alaninyl derivatives PH-223(*D*) (MIC:0.25-4 µg/ml) and PH-223(*L*) (MIC:0.25-2 µg/ml) showed potent activity against the four LNZ-resistant CNS-strains but were less active against the two LNZ-resistant *E. faecalis* strains with MIC value ranges of PH-223(*D*) (MIC:4-8 µg/ml) and PH-223(*L*) (MIC:4->16 µg/ml). Similarly, the 5-nitrothiophene-2-carbonyl substituted alaninyl

Table 6: Antibacterial activities of glycinyl and novel *N*-(*D*/L)-alaninyl oxazolidinones against susceptible and resistant bacterial strains

Compd code	MIC range (µg/ml) against													
	Enterococcus faecalis				*MRSA*		*CNS*						*Strep. pneum*[a]	
	ATCC 29212	Sus (n=7)	10** E*16µg	75** E*48µg	ATCC 25923	Sus (n=10)	ATCC 12228	Sus (n=10)	918** E* 48µg	919** E* 32µg	929** E* >256µg	941** E* 32µg	ATCC 49619	Sus (n=6)
PH214(D)	2	2	>16	>16	2	2	1	1	>16	>16	>16	>16	2	2
PH214(L)	2	2	8	8	2	2	2	2	4	4	4	4	2	2
PH218(D)	2	2-4	>16	>16	4	2-4	2	2	16	16	>16	16	2	1-2
PH219(D)	2	2-4	16	16	4	4	8	4	16	16	>16	>16	2	1
PH220(D)	2	2	16	16	2	2	1	1	4	4	>16	4	1	2
PH227(L)	1	1	16	16	1	1	1	1	2	4	8	4	1	1
PH228(L)	2	2-4	>16	>16	4	2-4	2	2	8	8	>16	8	2	2
PH229(L)	2	2-4	>16	>16	4	2-4	2	2	4	4	>16	8	1	1
PH223(D)	2	2	8	4	2	0.5-2	2	0.25	0.25	0.25	4	0.5	2	2
PH223(L)	2	2	>16	4	2	0.5-2	0.25	0.25	0.25	0.25	2	0.5	2	1-2
PH224(D)	2	2-8	>16	>16	2	2	0.5	0.5	0.5	0.5	16	1	2	1-2
PH232(L)	2	2	>16	16	2	2	0.5	0.5-1	0.5	0.5	2	2	1	1-2
PH145	< 0.5	< 0.5	2	1	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	1	< 0.5	< 0.5	< 0.5
PH189	< 0.5	< 0.5-2	4	2	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	1	< 0.5	< 0.5	< 0.5
LNZ	2	2	16	16	2	2	2	2	>16	>16	>16	>16	2	2

*E-test; **Linezolid-resistant. [a]*Streptococcus pneumonia*

derivatives PH-224(*D*) with MIC of 0.5-16 µg/ml and PH-232(*L*) with MIC of 0.5-2 µg/ml were active against 4 LNZ-resistant CNS strains, respectively, but inactive against the two LNZ-resistant *E. faecalis* strains with MIC value ranges of 16->16 µg/ml. However, the 5-nitro-2-furoyl glycinyl derivatives PH-145 (MIC: <0.5-1µg/ml) and PH-189 (MIC: <0.5-1) showed the most potent activity against all four LNZ-resistant *CNS* strains with MIC value ranges of <0.5-1µg/ml and <0.5-1), respectively. Furthermore, PH-145 and PH-189 also demonstrated activity against the two LNZ-resistant *E. faecalis* strains, with MIC value ranges of 1-2µg/ml and 2-4µg/ml, respectively [Table 6]. The non-heteroaroyl alaninyl derivatives were found to be generally less active than the heteroaroyl derivatives against all four LNZ-resistant CNS-strains, except for the 3,5-dinitrobenzoyl (PH-214(*L*), MIC:4µg/ml), 3-nitrobenzoyl (PH-220(*D*), MIC:4->16µg/ml), 3-nitrobenzoyl ((PH-220(*D*), MIC:4->16µg/ml and PH-227(*L*), MIC:2-8µg/ml) and 2-nitrobenzoyl (PH-229(*L*), MIC:4->16) derivatives [Table 6]. Both the *N*-substituted-(*D*/*L*)-alaninyl and *N*-substituted-glycinyl oxazolidinone derivatives evaluated in the study demonstrated superior activity against LNZ-resistant clinical isolates in comparison to linezolid.

CONCLUSION

The 15 N-substituted-(D/L)-alaninyl triazolyl oxazolidinones showed potent *in vitro* activity against M. tuberculosis M.tb H37Rv evaluated in the study. Of these, the seven most active derivatives were found to demonstrate moderate to potent anti-mycobacterial activity under aerobic and anaerobic conditions. Furthermore, these seven compounds showed moderate to strong activity against resistant strains and 4 of these demonstrated bactericidal activity. However, the 5-nitrothiophene-2-carbonyl derivatives PH-224(D) and PH-232(L) demonstrated activity against intracellular M. tuberculosis bacteria and were found to be cytotoxic. The N-substituted-amino acid triazolyl-oxazolidinone derivatives showed superior antibacterial activity than linezolid against most of the susceptible and clinical resistant Gram-positive cocci evaluated in this study, including LNZ-resistant strains. Compounds with high bactericidal activity and no cytotoxicity should be considered for further in vivo testing.

MATERIALS AND METHODS

Materials

The 15 compounds evaluated in this study were previously reported from our laboratory and were synthesized according to the literature methods [17, 27, 29]. The tested compounds are coded as follows PH-145, PH-189, PH-214, PH-215, PH-217, PH-218, PH-219, PH-220, PH-224, PH-227, PH-228 PH-229, PH-230, PH-231 and PH-232 (Figure 1 and Table 1). The compounds were purified either by silica gel column chromatography and / or recrystallization from suitable organic solvents or mixture of solvents. Column chromatography was performed using silica gel (Kieselgel 60, 70-230 mesh; Sigma-Aldrich) and TLC was carried out on 0.25 mm pre-coated silica gel plates ($60F_{254}$, Merck). All the compounds were fully characterized by spectrometric methods, including nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) spectroscopy on Bruker Avance II 600 NMR spectrometer and infra-red (IR) spectrophotometry on JASCO FT-IR-6300 (JASCO, Japan) spectrometer. Mass spectra were recorded on a Thermo Scientific DFS High Resolution Gas Chromatography / Mass Spectrometer (DFS GC-MS) and Quattro LC (Micro Mass, UK) Mass Spectrometer. Melting points determination were performed on a Stuart Scientific melting point apparatus (SMP1, UK). CHN elemental analyses were performed on an Elementar Vario Micro Cube CHN Analyzer apparatus (Elementar, Germany) and within ± 0.4% of the theoretical values. All analyses were performed at the Research Sector, General Facilities Science (GF-S), Faculty of Science, and Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, Kuwait.

Methods

Antimycobacterial susceptibility testing of M. tuberculosis

(Mtb) H37Rv and resistant strains, Mycobacterium abscessus subsp. bollettii 103 and Mycobacterium avium subsp. avium 2285 (S).

The antimycobacterial susceptibility testing using the M. tuberculosis (Mtb) H37Rv was performed by the National Institute of Health / National Institute of Allergy and Infectious Disease (NIH/NIAID, USA).

MIC determination against Mycobacterium tuberculosis H37Rv under aerobic conditions: The antimycobacterial activity of compounds against *Mycobacterium tuberculosis* H37Rv grown under aerobic conditions was assessed by determining the minimum inhibitory concentration (MIC) of compound. The MIC represents the lowest concentration (μM) of drug that visually inhibits growth of the microorganism. This assay is based on measurement of growth in a liquid medium of a fluorescent reporter strain of H37Rv where the readout is either optical density (OD) or fluorescence [32-36]. The MIC of compound was determined by measuring bacterial growth after 5 days in the presence of test compounds. Compounds were prepared as 20-point two-fold serial dilutions in DMSO and diluted into 7H9-Tw-OADC (4.7 g/L 7H9 base broth, 0.05% w/v Tween 80, 10% v/v OADC supplement) medium (Middlebrook 7H9 Base and Middlebrook OADC Supplement) in 96-well plates with a final DMSO concentration of 2%. The highest concentration of compound was 200 μM where compounds were soluble in DMSO at 10 mM. Each plate included assay controls for background (medium/DMSO only, no bacterial cells), zero growth (2 μM rifampicin) and maximum growth (DMSO only), as well as a rifampicin dose response curve. Plates were inoculated with *M. tuberculosis* and incubated for 5 days: growth was measured by OD_{590} and fluorescence (Ex 560/Em 590) using a BioTek™ Synergy H4 plate reader. Growth was calculated separately for OD_{590} and RFU. The MIC was calculated from the dose response curve and was plotted as % growth and fitted to the Gompertz model using Graph Pad Prism 6. The MIC was defined as the minimum concentration at which growth was completely inhibited and was calculated from the inflection point of the fitted curve to the lower asymptote. In addition, dose response curves were generated using the Levenberg-Marquardt algorithm and the concentrations that resulted in 50% and 90% inhibition of growth were determined (IC_{50} and IC_{90}) respectively.

MIC determination against Mycobacterium abscessus subsp. bollettii 103 under aerobic conditions [32,33].: The antimycobacterial activity of compounds against *Mycobacterium abscessus* subsp. bollettii 103 was assessed by inoculating plates with *M. abscessus*, the plates were incubated for 3 days at 37˚C and the growth was measured by OD_{590}. The dose response curve was plotted as % growth and fitted to the Gompertz model. The MIC was defined as the minimum concentration at which growth was completely inhibited and was calculated from the inflection point of the fitted curve to the lower asymptote. MIC values were reported when the following quality control criteria were satisfied: Firstly, for each plate, no growths in the background (un-inoculated) control wells and OD_{590} >0.2 in maximum growth wells. Secondly, for each compound curve, MIC values were reported if there were 2 points with growth >75% and if there were 2 points with growth <75%. Finally, if no point reached 75% inhibition, the MIC was reported as > maximum concentration tested.

MIC determination against Mycobacterium avium subsp. avium 2285 (S) under aerobic conditions [32-34]: The antimycobacterial activity of compounds against *M.* avium subsp. avium 2285 (S) was assessed by inoculating plates with *M. avium* and the plates were incubated for 5 days at 37˚C and Alamar blue was added to each well (10 μL of Alamar blue to 100 μL culture) and incubated for 24 h at 37˚C. Plates were visually inspected and the color recorded for each well. The MIC was defined as the lowest concentration at which no metabolic activity was seen (blue well). MIC values were reported when the following quality control criteria were satisfied: For each plate, the background (un-inoculated) control wells remain blue, maximum growth wells are pink and inhibition control wells are blue. Also, for each compound, MIC values were reported if there was a transition from pink to blue. Finally, if all wells were pink, the MIC was reported as > maximum concentration tested.

MIC determination under low oxygen conditions: Traditional screening of compounds for activity against *M. tuberculosis* usually targets the organism in an active replicating state. However, since *Mtb* can reside in a state of non-replicating persistence (NRP), which may represent low-oxygen recovery stage hence the need for this assay. The micro plates were prepared for this assay according to literature procedures [37-39]. Test compounds were prepared as 20-point two-fold serial dilutions in DMSO and diluted into Dubos-Tw-Albumin DTA: 6.5 g/L Dubos broth base, 10% (v/v) soluble in DMSO at 10 mM. For compounds with limited solubility, the highest concentration was 50x less than the stock concentration of 100 μM for 5 mM DMSO stock, 20 μM for 1 mM Dubos medium albumin, 0.05% w/v Tween 80 medium in 96-well plates with a final DMSO concentration of 2%. The highest concentration of compound was 200 μM where compounds were DMSO stock. Control compounds were prepared as two-fold serial dilutions in DMSO and diluted into DTA medium in 96-well plates with a final DMSO concentration of 2%. *M. tuberculosis* constitutively expressing the *luxABCDE* operon was inoculated into DTA medium in gas-impermeable glass tubes and incubated for 18 days to generate hypoxic conditions (Wayne model of hypoxia) [37]. At this point, bacteria are in a non-replicating state (NRP stage 2) induced by oxygen depletion. Oxygen-deprived bacteria were inoculated into compound assay plates and incubated under anaerobic conditions for 10 days followed by incubation under aerobic conditions (outgrowth) for 28h, and growth was measured by luminescence. Oxygen-deprived bacteria were also inoculated into compound assay plates and incubated under aerobic conditions for 6 days, and growth was measured by luminescence. Rifampicin was included in each plate and metronidazole was included in each run as positive controls for aerobic and anaerobic killing of *M. tuberculosis*, respectively.

Minimum Bactericidal Concentration (MBC) Assay: The minimum inhibitory concentration was determined as follows, *M. tuberculosis* was grown aerobically to logarithmic phase on Middlebrook 7H10 agar plates and inoculated into liquid medium containing four different compound concentrations [40] with a final maximum concentration of 2% DMSO. For compounds with an MIC <20 μM (from Task Group 1 assay), the concentrations selected were 10X MIC, 5X MIC, 1X MIC, and 0.25X MIC. The cultures were exposed to compounds for 21 days and cell viability measured by enumerating colony forming units on agar plates on day 0, 7, 14 and 21. MBC was defined as the minimum concentration required to achieve a 2-log kill in 21 days. For compounds with >1-log kill, an assessment of time- and/or concentration-dependence was determined from the kill kinetics. DMSO was used as a positive control for growth.

Intracellular (Macrophage) and Cytotoxicity Screening Assay [39]

Intracellular Activity Assay: The activity of compounds against intracellular bacteria was determined by measuring viability in infected THP-1 cells after 3 days in the presence of test compounds. Compounds were prepared as 10-point serial dilutions in DMSO. The highest concentration of compound tested was 50 μM where compounds were soluble in DMSO at 10 mM. THP-1 cells were cultured in complete RPMI and differentiated into macrophage-like cells using 80 nM PMA overnight at 37°C, 5% CO_2. THP-1 cells were infected with a luminescent strain of Mtb H37Rv (which constitutively expresses *luxABCDE*) at a multiplicity of infection of 1 and incubated overnight at 37°C, 5% CO_2. Infected cells were recovered using Accutase/EDTA solution, washed twice with PBS to remove extracellular bacteria, and seeded into assay plates. Compound dilutions were added to a final DMSO concentration of 0.5%. Assay plates were incubated for 72 h at 37°C, 5% CO_2. Each run included isoniazid as a control. Relative luminescent units (RLU) were measured using a Biotek Synergy 2 plate reader. The dose response curve was fitted using the Levenberg-Marquardt algorithm. The IC_{50} and IC_{90} were defined as the compound concentrations that produced 50% and 90 % inhibition of bacterial growth respectively.

Cytotoxicity Assay

The cytotoxicity of compounds was determined by measuring THP-1 cell viability after 3 days in the presence of test compounds. Compounds were prepared as 10-point serial dilutions in DMSO. The highest concentration of tested compound was 50 μM where compounds were soluble in DMSO at 10 mM. THP-1 cells were cultured in complete RPMI and differentiated into macrophage-like cells using 80 nM PMA overnight at 37°C, 5% CO_2. Cells were inoculated into assay plates and cultured for 24h before compound dilutions were added to a final DMSO concentration of 0.5%. Each run included staurosporine as a control. Assay plates were incubated for 3 days at 37°C, 5% CO_2; growth was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) which uses ATP as an indicator of cell viability. Relative luminescent units (RLU) were measured using a Biotek Synergy 4 plate reader. The dose response curve was fitted using the Levenberg–Marquardt algorithm. The IC_{50} was defined as the compound concentration that resulted in 50% viability.

Antibacterial susceptibility testing of linezolid-resistant Gram-positive cocci: Antibacterial susceptibility testing for compounds was performed by determining the minimum inhibitory concentrations (MIC's, μg/ml), which is defined as the lowest concentration of a compound that inhibits visible bacterial growth. The MICs were determined on Mueller Hinton (MH) agar according to the Clinical and Laboratory Standard Institute (CLSI) [40] with medium containing dilutions of the compounds ranging from 0.12 to 16 μg/ml. The reference drug linezolid was dissolved in 40% water in ethanol while all other test compounds were dissolved in 80% DMSO in water. MH agar plates were used for all staphylococci and enterococci, and MH agar plates were supplemented with 5% sheep blood to facilitate the growth of *S. pneumoniae*. The Gram-positive clinical isolates at the MRSA Reference Laboratory, Faculty of Medicine, Kuwait University utilized in this study consisted of linezolid-susceptible Methicillin-resistant *S. aureus* (MRSA, n = 10), linezolid-susceptible Methicillin-resistant coagulase-negative staphylococci (MR-CNS, n = 6), LNZ-resistant MR-CNS (n = 4), linezolid-susceptible *Enterococcus faecalis* (n = 7), linezolid-resistant *E. faecalis* (n = 2) and Penicillin-resistant *S. pneumoniae* (n = 10). E test® reagent strips were used to identify resistant microorganisms [40]. Standard reference strains used included *S. aureus* ATCC25923, *CNS* and *S. epidermidis* ATCC12228, *E. faecalis* ATCC29212, *S. pneumoniae* ATCC49619. The final bacterial concentration for inoculum was 10^7 CFU/ml and was incubated at 35 °C for 18 h.

Consent for Publication: Not applicable.

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Authors' Contributions

Professor Oludotun A. Phillips (OAP) and Dr. Edet E. Udo (EEU) jointly conceived the idea and designed the experiments; chemical synthesis of compounds was performed by VT and LHS in the laboratory of OAP; microbiology evaluation against Gram-positive cocci was performed by LHS and TV in the laboratories of EEU. The ant tuberculosis testing was performed by National Institute of Health / National Institute of Allergy and Infectious Disease (NIH/NIAID). OAP and LHS jointly analyzed data and wrote the manuscript. The manuscript was reviewed by all the contributors.

Conflict Of Interest

The authors declare no conflict of interest. We also declare

that the funding source was not involved in study design, in collection, analysis and interpretation of data, in writing of the report; and in the decision to submit the article for publication.

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