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6 Nitro-1-benzylquinolones exhibiting specific anti-tubercular activity

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Abstract

In this study, we synthesized novel nitro quinolone-based compounds and tested them *in vitro* against a panel of Gram-positive and Gram-negative pathogens including *Mycobacterium tuberculosis* (MTB), *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Escherichia coli* for antibacterial activities and also against HeLa cells for overt cytotoxicity. Compound **8e** was identified as a non-toxic, potent hit with selective activity (MIC_{90} <0.24 μM) against MTB. **8e**, however, showed no activity against DprE1 mutant, suggesting DprE1 as the likely target for this compound class.

Keywords: Quinolones, *Mycobacterium tuberculosis*, Nitro drugs, DprE1 enzyme.

1. Introduction

Although the genome of *Mycobacterium tuberculosis* (MTB), the causal agent of tuberculosis (TB), has been completely sequenced and several drug targets identified (Cole et al., 1988), potent inhibitors of many targets remain scarce. In part, this is because compounds identified *via* target-based screening often fail to exhibit activity when tested against whole-cell MTB (Jansen and Rhee, 2017). The notorious structural and pathophysiological features of MTB represent a major confounder, precluding inhibitors from reaching their targets (Kana et al., 2014). For-example, MTB possesses a cell wall core made up of mycolic acids esterified with arabinogalactan, which in turn is covalently attached to peptidoglycan. This core is interspersed by various lipids, proteins, and carbohydrates (Brennan, 2003). Underneath this core lies the usual lipid bilayer ─ the cell membrane, which is found in all bacteria (gram +ve, and -ve inclusive) (Barak and Muchova, 2013). All these collectively form an impermeable barrier against potential drug molecules. In addition, a definitive pathophysiological hallmark of active MTB infection is the formation of granulomas, which result from inflammatory response to MTB. In the simplest description, the granuloma consists of MTB-containing macrophages, other immune cells, and dead cells, and is devoid of blood vessels (Warsinske et al., 2017). These effectively shield MTB from the effect of xenobiotics *in vivo* (Warsinske et al., 2017). Hence, in contrast to target-based drug discovery, whole cell-based screens can be used to identify compounds able to penetrate MTB and affect its viability (Kana et al., 2014).

It is estimated that approximately one-quarter of the world's population harbour MTB in its latent form, and almost 10 % of this cohort will develop active pulmonary TB in their life time (Gideon and Flynn, 2011). At least 10 million active pulmonary TB cases and 1.6 million TB related deaths were reported in 2018 (WHO, 2018). TB is a public health emergency owing to the existence of several forms of drug(s) resistant MTB, including rifampicin resistance (RR), isoniazid resistance (IR), multidrug resistance (MDR), and extensively drug resistance (XDR) (WHO, 2018), all of which are transmissible (Leung et al., 2013). Increasing prevalence of drug-resistant MTB places current TB treatments in jeopardy, and makes paramount the search for new compounds with novel modes of action against the bacillus. Recently, a new regimen (comprising bedaquiline, pretomanid, and linezolid) was approved by the US Food and Drug Administration (FDA) (FDA, 2019). However, while very exciting, this success is inadequate given the global burden of drug resistant MTB. In

addition to the safety concerns, other alternatives are needed to avoid over prescription of the new regimen and to slow the emergence of drug-resistance.

Nitro benzenoids (see **1-2, Fig. 1**) have recently been identified as new compounds showing activity against both drug-susceptible and drug-resistant forms of MTB (Chikhale et al., 2018). They exhibit potent inhibitory activity through a mode of action (MoA) that differs from that of anti-tubercular agents in clinics. Most notably, the leading compound from this class of compounds, PBTZ169, is reported to act as suicide inhibitor of the mycobacterial decaprenylphosphoryl-β-D-ribose 2′-epimerase (DprE1) (Makarov et al., 2014). DprE1 is required for the synthesis of arabinogalactan, an essential component of the mycobacterial cell wall (Chikhale et al., 2018). Leads in this compound class have interesting attributes: they are pro-drugs activated by an enzyme peculiar to MTB, hence reducing the probability of side effects (Trefzer et al., 2010). However, they also suffer from poor drug-like properties such as short half-life and poor aqueous solubility (Zhang et al., 2019). These warrant further research in this area.

The quinolone class of antibiotics – which are characterized by carboxyl and amino moieties attached at positions -3 and -7, respectively, of the quinolone nucleus and generally referred to as fluoroquinolones (FQ) – has long served as mainstay antibiotic for a wide range of bacterial infections, including TB (Asif, 2013). Their MoA involves targeting bacterial DNA gyrase and/or topoisomerase IV (Aldred et al., 2014). The FDA currently recommends a black box label for all fluoroquinolones (FDA, 2019b). While a structural alteration strategy could be used to mitigate off-target effects of drugs (Kerns and Carter, 2009), this is difficult with the fluoroquinolones as the very structural features that promote antibacterial activities are also implicated in off-target activities. For-example, the amine moiety at position -7 of the quinolone nucleus which is crucial for antibacterial activity has been reported to also promote genotoxicity and inhibition of GABA receptors (Pham et al., 2019). Three studies have investigated 6- nitro-7-aminoquinolone-3-carboxylic acid for anti-TB activity (Senthilkumar et al., 2009; Artico et al., 1999; Sbardella et al., 2004). The most active compound from these studies (**3, Fig. 1**) shares significant structural similarity with ciprofloxacin (**4, Fig. 1**), a FQ (Senthilkumar et al., 2009), and exhibits reduced activity against FQ resistant-TB (0.16 μM), which indicates cross resistance with fluoroquinolones (Beteck et al., 2018). Compounds in

these studies were also active against Gram-positive and Gram-negative bacteria just like FQ (Artico et al., 1999; Sbardella et al., 2004).

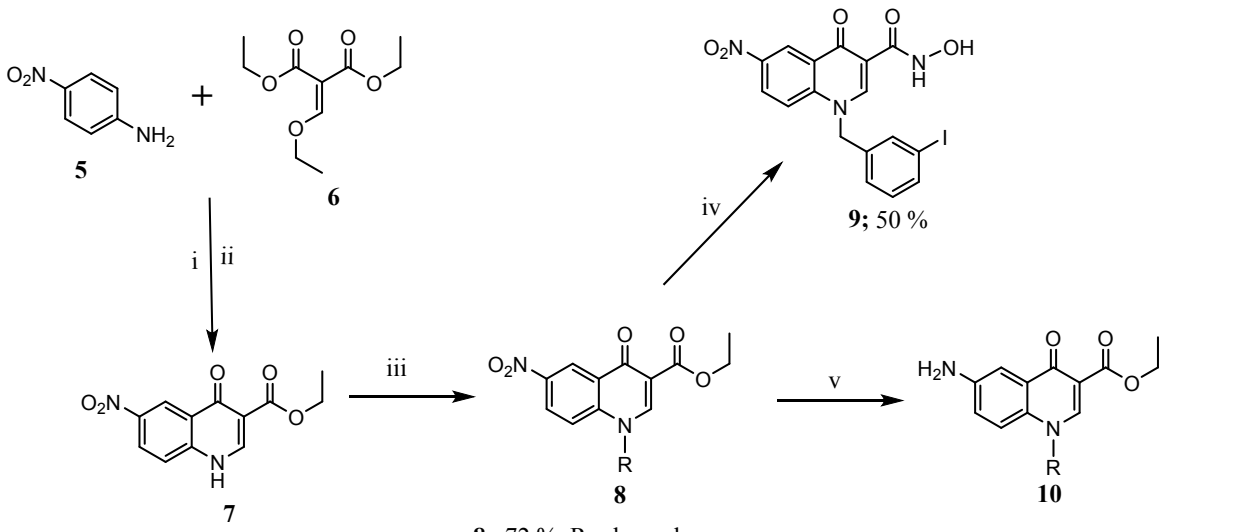
Besides MTB, a good number of bacteria collectively referred to as "ESKAPE" pathogens are of global public health concern because they have developed resistance against all clinically available antibiotics and, more importantly, because these pathogens are responsible for healthcare-associated infections (HAI) (Mulani et al., 2019). HAI are difficult to treat, necessitating longer stays in the hospital, which ultimately increases treatment cost (Mulani et al., 2019). To this effect, we cross screened compounds investigated for anti-tubercular activity against some ESKAPE pathogens in order to identifying new hit compounds against these pathogens.

In this study, we synthesized novel 6-nitroquinolones and evaluated them *in vitro* for anti-tubercular and antibacterial activities. Target compounds were achieved through simple synthetic transformations illustrated in **scheme 1**, below. Briefly, a nucleophilic conjugate addition reaction between **5** and **6** gave a condensed intermediate which underwent intramolecular Friedle-Craft acylation reaction in Eaton's reagent at 90 ^{o}C to afford compound **7**. Treatment of **7** with various benzyl bromides using previously reported (Beteck et al., 2019) procedures effected *N*-alkylation of the secondary amine to furnish target compounds **8a-8j** in 70-85 % overall yields. Treatment of **8b** in refluxing hydroxylamine solution (50 % wt in H_2O) effected aminolysis of the ethyl ester to afford compound **9**, which is a hydroxamate, in 50 % yield. The NO_2- group in compounds **8b-8h** and **8j** was selectively reduced to an NH_2- moiety using reduced iron powder and acetic acid. This transformation afforded compound **10a-10h** in 50-60 % yield.

All target compounds (**8a-8j, 9, 10a-10h**) were characterized using ^{1}H and ^{13}C NMR, and HRMS. For compound **8a-8j,** and **10a-10h**, the ^{1}H NMR spectra show a singlet peak appearing at *ca* 5.7 ppm which is assignable to the $-CH_2-$ of benzyl substituents. The quartet peak (J = 7.1) appearing at *ca* 4.3 ppm is attributable to the $-CH_2-$ of ethyl ester, while the triplet signal (J = 7.1) at *ca* 1.3 ppm indicates the presence of the $-CH_3$ of ethyl ester. The disappearance of the peaks at *ca* 4.3 and 1.3 ppm, and the appearance of singlet at 11.6 ppm (which is absent in **8b**) in the ^{1}H NMR spectrum of compound **9** suggests that the ethyl ester was successfully transformed to a hydroxamate. The appearance of a singlet peak at *ca* 5.4 ppm in the ^{1}H NMR spectra of compound **10a-10h** is indicative

of the presence of aryl-NH_2; this peak is absent in the spectra of the precursor compounds (**8a-8j**). High-resolution mass spectrometry analysis further confirmed the desired molecular ions, which were consistent with structures of target compounds.

Scheme 1. Synthesis of target compounds[a]



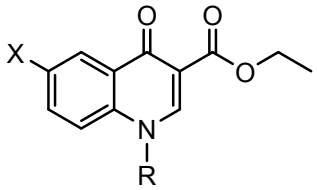
8a;72 %, R = benzyl
8b;70 %, R = 3-I-benzyl
8c;78 %, R = 4-Br-benzyl
8d;80 %, R = 3-Br-benzyl
8e;73 %, R = 3-Cl-benzyl
8f;76 %, R = 4-F-benzyl
8g;77 %, R = 3-F-benzyl
8h;70 %, R = 4-CN-benzyl
8i;72 %, R = 3-CN-benzyl
8j;80 %, R = 3-CH_3-benzyl

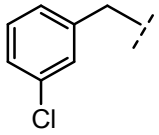
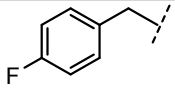
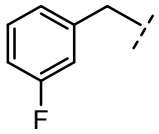
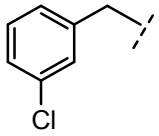
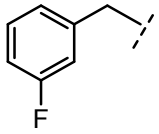
10a;60 %, R = 3-I-benzyl
10b;52 %, R = 4-Br-benzyl
10c;55 %, R = 3-Br-benzyl
10d;58 %, R = 3-Cl-benzyl
10e;50 %, R = 4-F-benzyl
10f;50 %, R = 3-F-benzyl
10g;56 %, R = 4-CN-benzyl
10h;57 %, R = 3-CH_3-benzyl

[a]**Reagents and conditions**. (i) EtOH, reflux, 2-3 hours, (ii) Eaton's reagent, N_2 atmosphere, 80-90 ^oC, 12-15 hours, (iii) Benzyl bromide, K_2CO_3, DMF, reflux, 10-12 hours, (iv) NH_2OH (50% wt in H_2O) (v) Fe/Acetic acid, EtOH, ultrasound irradiation, 3-6 hours.

All target compounds were screened *in vitro* against *Mtb::gfp* reporter strain cultured in Middlebrook 7H9 media supplemented with casitone, glucose and tyloxpol for anti-tubercular evaluation. Each compound was screened at ten different concentration points (0.0002 – 10mM) and a dose-response curve was generated at day 14, was constructed from which the MIC_{90} values were determined. The MIC_{90} value refers to the minimum concentration of a test compound required to inhibit 90 % of MTB growth. Rifampicin was included as the positive control in this assay. Five out of the nineteen compounds inhibited MTB growth, demonstrating anti-MTB activity in the range of 0.24 to 62.5 μM. Compound **8e** exhibiting an MIC_{90} value of <0.24 μM emerged as the most active compound in this study. Although the number of compounds showing anti-MTB activity in this study was relatively small, precluding extensive structure activity relationship (SAR) analysis, comparison of compounds showing anti-mycobacterial activity suggests that *meta* substituted benzyl moieties at position -1 of the quinolone nucleus promote activity better than *para* substituted benzyl moieties. For example, compound **8f** (MIC_{90}: 8.4 μM) bearing a *para* fluoro benzyl moiety at position -1 is two-fold less active than compound **8g** (MIC_{90}: 4.1 μM) having a *meta* fluoro benzyl moiety at this position (see **Table 1**). Moreover, comparing compound **8f** (MIC_{90}: 8.4 μM) against **10e** (MIC_{90}: >125 μM), compound **8g** (MIC_{90}: 4.1 μM) against **10f** (MIC_{90}: 15.4 μM), and compound **8e** (MIC_{90}: 0.24 μM) against **10d** (MIC_{90}: 62.5 μM), shows that anti-MTB activity decreases multiple fold when the NO_2- moiety is replaced by an NH_2- moiety. This suggests that the nitro group promotes anti-TB activity than amino group─more analogues will be exploited to further confirm this.

Table 1. Structural features, and anti-MTB activity of selected hit compounds compared against rifampicin.



#[a]	X	R	MTB MIC_{90} (μM)	MW[b]	XlogP3[c]	R5V[d]	PA[e]	P-gp substrate?[f]
8e	NO_2		0.24	386	4.0	none	none	no
8f	NO_2		8.4	370	3.5	none	none	no
8g	NO_2		4.1	370	3.5	none	none	no
10d	NH_2		62.5	356	3.5	none	none	no
10f	NH_2		15.4	340	3.0	none	none	no
Rif	-	-	0.010	-	-	-	-	-

[a]: # = compound number. [b]: Molecular weight (MW) generated using chem draw professional version 12. [c]: XlogP3, calculated lipophilicity. [d]: R5V = Lipinski's rule of five violation. [e]: PA = PAIN Alert. [f]: P-gp = P-glycoprotein. [c,d,e,f] were predicted using SwissADME website . Rif = Rifampicin.

It is paramount to note that antibacterials (antimicrobials) are pivotal to the practice of modern medicine, wherein they are deployed in surgery, organ(s) transplant, radiotherapy, sterilization, medical implants, treatment and management of wounds and various types of infections (Yang et al., 2018). The years 1940-1960 referred to as the "golden age of antibiotics" saw an increase in the discovery and development of several antibiotics, although centered around a few compound classes including fluoroquinolones, *β*- lactams, aminoglycosides, tetracyclines, and macrolides (Davies, 2006). With the emergence and spread of several forms of drug resistant bacteria, and pharmaceutical companies opting out of antibacterial research (Pendleton et al., 2013), the practice of modern medicine in the near future will face several limitations if new antibacterials are not identified and developed. To this effect, all target compounds were also evaluated *in vitro* against five other bacteria (*E. coli*, *A. baumanni*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*) for antibacterial activities (Blaskovich et al., 2015). Bacteria were cultured in Cation-adjusted Mueller Hinton broth (CAMHB). Colistin and vancomycin were used as standards for Gram-negative and Gram-positive bacteria, respectively. At a single point concentration of 32 μg/ml, none of the compounds tested inhibited bacterial growth above 50 % and, hence, were considered inactive against these bacteria. This result suggests that the hit compounds exhibit selective anti-MTB activity probably through modulating a target that is unique/vital only to MTB. It is important to mention that compounds exhibiting selective anti-microbial activity are likely to have a large safety margin.

Since hit compounds showed specific antitubercular activity which seems to be depended on the presence of the 6-nitro moiety, compound **8e** was screened against DprE1 mutant to establish possible mode of action. **8e**, however, showed no activity (MIC_{90}: >125 μM) against DprE1 mutant, suggesting DprE1 as the likely target for this compound class. The binding mode of **8e** and its amino derivative (**10d**) with the crystal structure of DprE1 enzyme were studied using Glide Ligand Docking as implemented in Maestro in the Schrödinger package. The enzyme crystal structure (PDB: 4KW5) was obtained from protein data bank (PDB). Both compound **8e** and **10d** showed good docking scores of -6.267 and -5.579, respectively; which indicates binding with the enzyme. The Protein-ligand interactions of both compounds shows the presence of intramolecular hydrogen bonding which are essential for locking the ligand in the active site, and therefore a theoretical indication of inhibition. **8e** interacted with the enzyme through *pi-pi* stacking with TRP230 and hydrogen bonding with ASN385.

10d interacted with the enzyme through *pi-pi* stacking with LYS418, halogen bonding with SER228 and hydrogen bonding with HIS132 and TYR60. The binding modes of 8e and 10d are presented in figure 2a and 2b below.

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Potential cytotoxicity of these compounds was assessed *in vitro* against HeLa cells cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza) using a resazurin cell viability assay. Emetine was included as a positive control. At 20 μM, none of the tested compounds inhibited HeLa cell viability to below 50 %. This result indicates that the compounds exhibit low cytotoxicity against human cells at 20 μM.

The drug-like properties of compounds showing anti-MTB activity were predicted online through the swissADME website. Calculated lipophilicity (ClogP) values for these compounds were ≥ 3, but not more than 5 (see **Table 1**), and their water solubility profiles were predicted to be very poor. These compounds were also predicted to have low risk of acting as pan-assay interference (PAIN) compounds ─ this is also evident in their selective anti-MTB activity. They all conform to Lipinski's rule of five, and are predicted not to act as substrate for P-glycoprotein. The aqueous solubilities of compounds **8e** and **8g** were determined experimentally in phosphate buffered saline at pH 7.4 to be < 5 μM, which is very poor. This result corroborated the solubility profiles predicted by swissADME website.

2. Conclusion

In conclusion, this study has generated novel nitroquinolone and aminoquinolone based compounds. The compounds were evaluated *in vitro* against a panel of bacteria, including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Staphylococcus aureus*, and *Escherichia coli* and five compounds were shown to exhibit specific anti-MTB activity. SAR analyses of these hits suggest that a nitro moiety at position 6 of the quinolone nucleus enhances anti-tubercular activity over an amino group─ more compounds are needed to further confirm this. Compound **8e**, the most active compound in this study, elicits a potent activity profile (MIC_{90}: <0.2μM). This hit compound was inactive against DprE1 mutant, which posits DprE1 as the most probable target. Compound **8e** also exhibited good docking score of -6.267 against DprE1 enzyme, which further confirms possible interaction with the enzyme. Moreover, this compound conforms to the Rule of Five and has no predicted PAIN liability structural features. **8e** however suffers from poor aqueous solubility (< 5 μM), an observation which motivates for further research in this area.

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Conflict of interest.

The authors have no conflict of interest to declare

Data Availability Statement

Detailed characterization data together with ^{1}H,^{13}C and HRMS spectra for compounds **8a-j**, **9** and **9a-h** are available in the supplementary file. Samples of compounds can be obtained from corresponding authors upon request.

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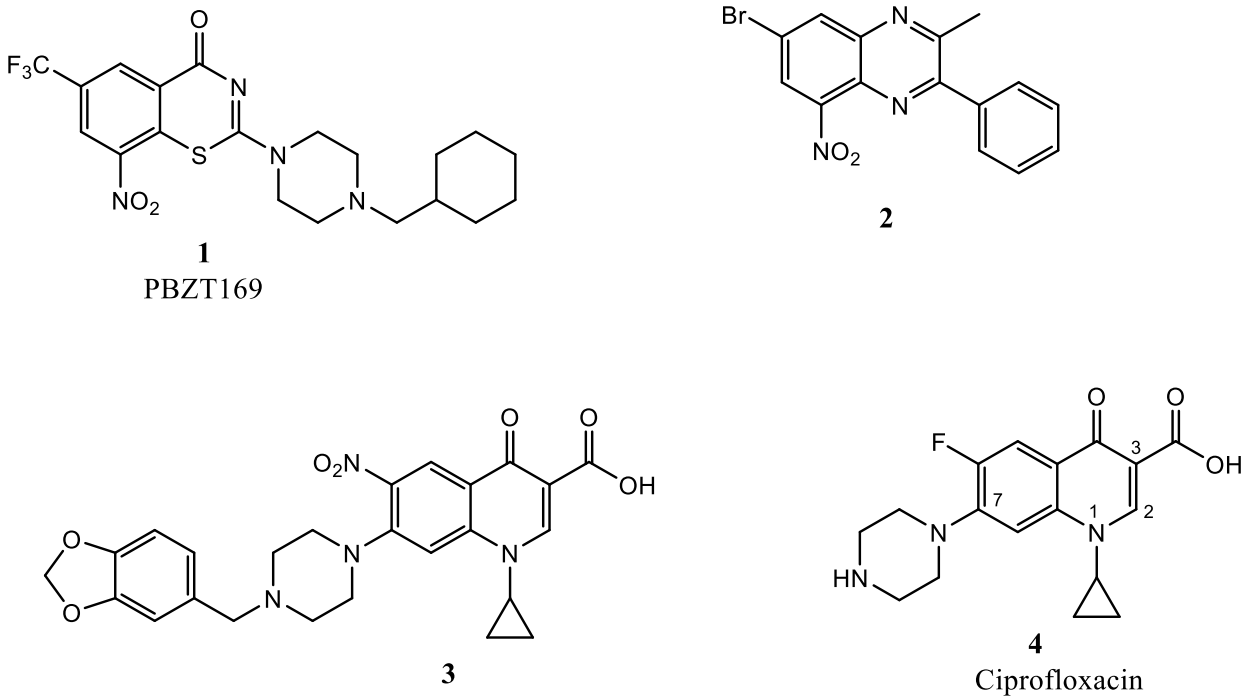

Figure 1. Structures of nitro benzenoids with anti-tubercular activity, and fluoroquinolone (**4**).

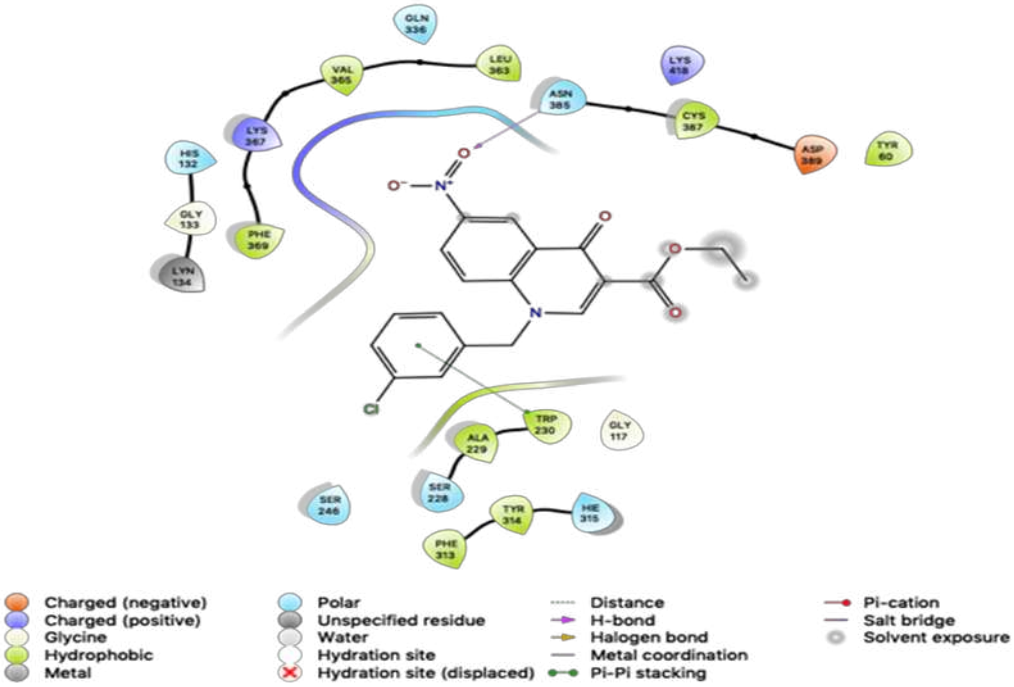

Figure 2a. Binding orientation of **8e** with DprE1 enzyme.

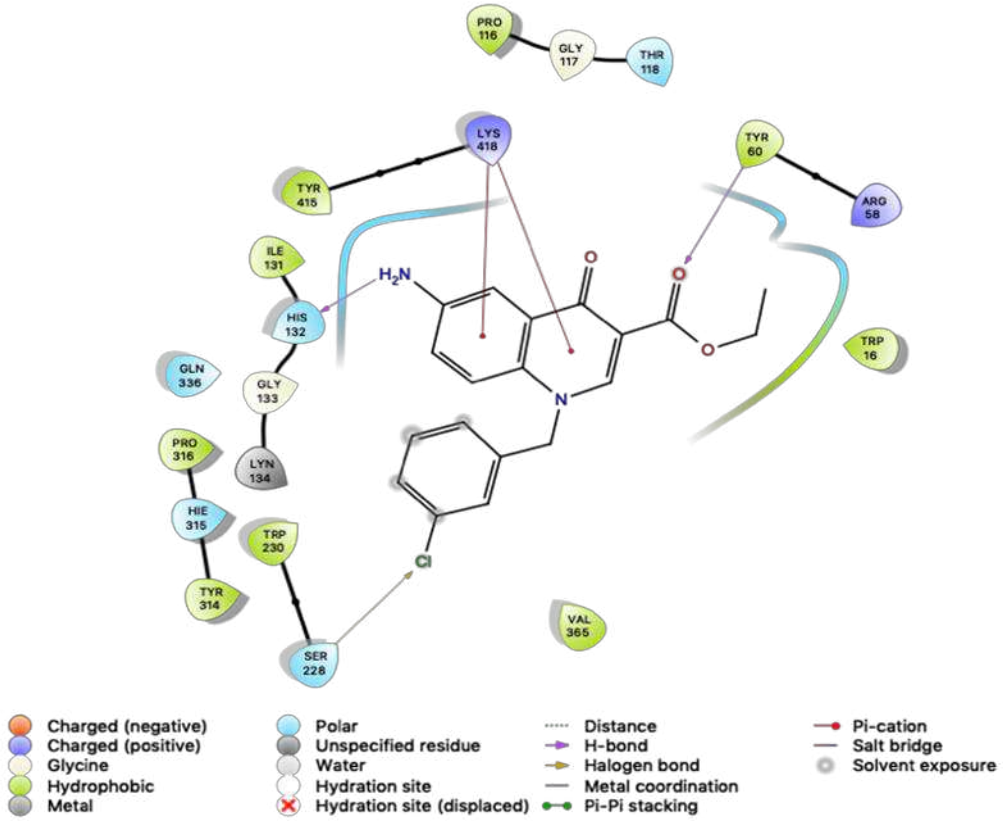

Figure 2b. Binding orientation of **10d** with DprE1 enzyme