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Original article

Synthesis and *in vitro* antimycobacterial activity of balofloxacin ethylene isatin derivatives

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ABSTRACT

A series of novel balofloxacin ethylene isatin derivatives with remarkable improvement in lipophilicity, as compared to the parent compound balofloxacin, were designed, synthesized and characterized by ^{1}H NMR, MS and HRMS. These derivatives were initially evaluated for their *in vitro* antimycobacterial activity against *M. phlei* CMCC 93201 and *M. smegmatis* CMCC 93202. Compounds **3b**, **3d**, **3g**–**j** and **3l** were chosen for further evaluation their *in vitro* activity against MTB 09710 clinical isolate, and then compounds **3h** and **3g** against MTB H37Rv ATCC 27294. All of the synthesized compounds were less active than balofloxacin against *M. phlei* CMCC 93201 and *M. smegmatis* CMCC 93202, but compounds **3g**–**j** (MIC: <0.5–8 μg/mL) were more potent than balofloxacin (MIC: 16 μg/mL) against MTB 09710. In particular, compound **3h** (MIC: 0.25– < 0.5 μg/mL) was found to be comparable to moxifloxacin, and ≥32 fold more potent than balofloxacin against MTB 09710 and MTB H37Rv ATCC 27294. The results demonstrated that the lipophilicity of the tested compounds was not the sole parameter affecting antimycobacterial activity, as well as the potential and importance of developing new fluoroquinolone derivatives against mycobacterial infections.

1. Introduction

Tuberculosis (TB), caused predominantly by *M. tuberculosis* (MTB), is one of the most common infectious diseases known to man. The WHO has estimated that 9.2 million new incident cases in 2006, with approximately 1.7 million individual deaths in the same year [1], and one-third of the world population (around 1.7 billion) is latently infected with MTB [2]. In India alone, one person dies of TB every minute [3]. The major concerns for current TB treatment are its latency, co-infection with HIV, poor patient compliance, and drug resistance issues caused by the emergence of multidrug resistant tuberculosis (MDR-TB) and the recent advent of extensively drug resistant tuberculosis (XDR-TB) [4,5]. It is estimated that between 2002 and 2020, approximately 1 billion people will be newly infected, more than 150 million people will get sick, and 36 million will die of TB if new disease prevention and treatment measures are not developed [6]. Hence, there is an urgent need to develop novel, high effective, and fast acting anti-TB drugs with low toxicity profiles and performing activity against both actively growing and latent infections [7].

Fluoroquinolones (FQs) exert their bactericidal activity by interfering with the function of two type II bacterial topoisomerase enzymes, DNA gyrase (the principal target in gram-negative bacteria) and topoisomerase IV (the main target in gram-positive bacteria) [8]. The incidence of mycobacterial resistance to FQs is relatively low at the present time, and there are no reports of cross-resistance or antagonism with other classes of anti-TB agents [9]. Several FQs, including ciprofloxacin, ofloxacin and sparfloxacin are currently recommended as second-line agents by WHO for the treatment of TB primarily in cases involving resistance or intolerance to first-line anti-TB therapy [10]. Interestingly, C-8 methoxy FQ derivatives with N1-cyclopropyl substitution are much more active against resistant MTB than C-8 hydrogen compounds [11]. For example, moxifloxacin (MXFX) and gatifloxacin (Fig. 1) were observed to have a particularly strong *in vitro* and *in vivo* activity (similar to isoniazid) against MTB and may be able to reduce the length of therapy owing to activity against both replicating and non-replicating bacilli [12,13].

Structure-activity relationship (SAR) studies of FQs indicated that the substituents at C-7 position greatly influence their potency, antibacterial spectrum as well as safety [14], and substitution of bulky functional group is permitted at the C-7 position [15]. Moreover, the lipophilicity of the FQs plays an important role in the penetration of these compounds into bacterial cells, and simply increasing the lipophilic character at C-7 position could also increase the anti-TB activity [16]. Therefore, reasonable modification at C-7 position is likely to provide more effective anti-TB agents.

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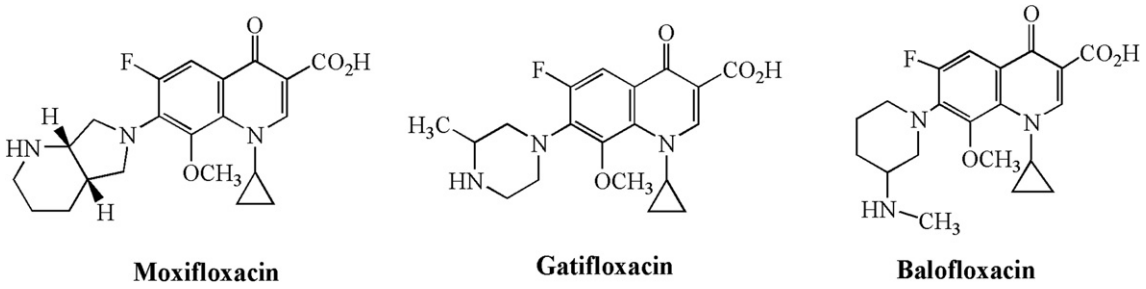


Fig. 1. Chemical structures of Moxifloxacin, Gatifloxacin and Balofloxacin.

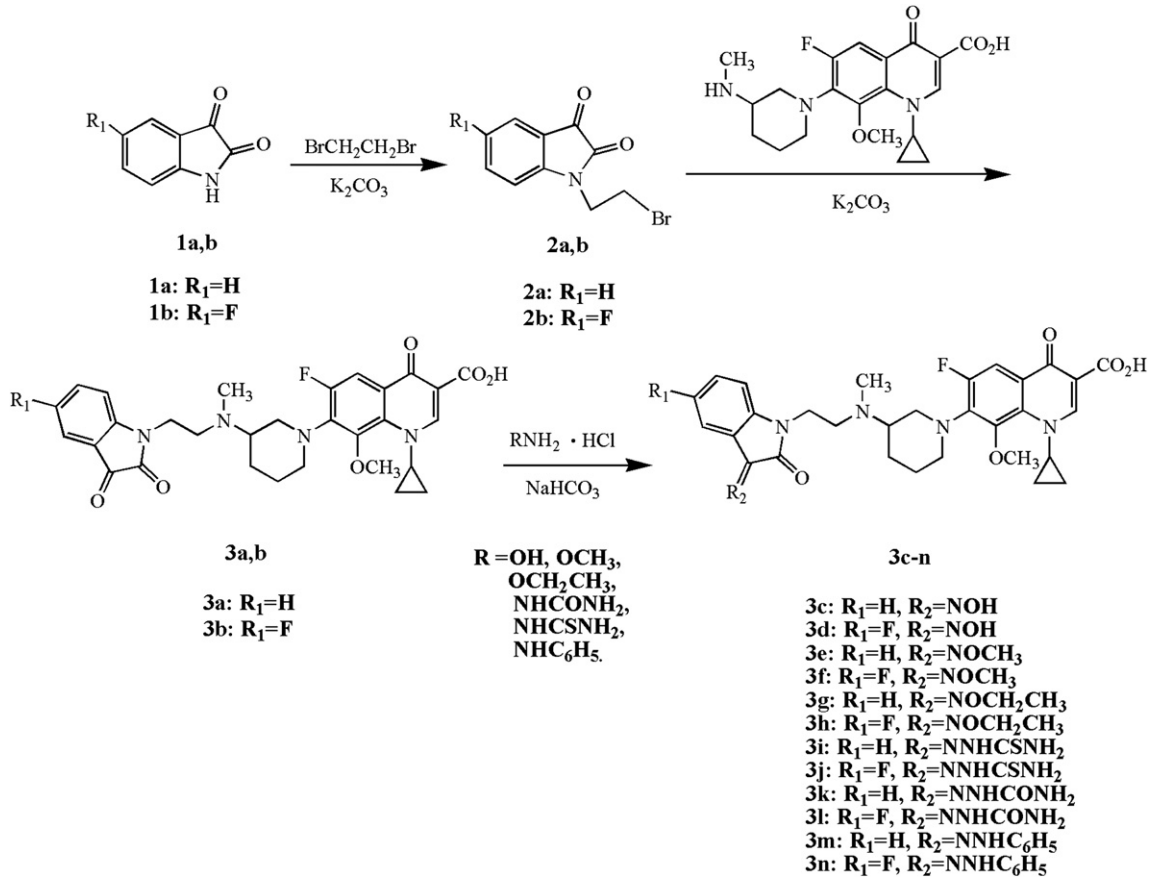
Balofloxacin (Fig. 1, BLFX), one of C-8 methoxy FQs with abroad spectrum against gram-positive and gram-negative bacteria, is comparable to that of ofloxacin or levofloxacin against MTB, *M. kansasii* and *M. fortuitum* [17]. However, to date few studies have been undertaken to optimize BLFX against mycobacteria.

Recently, as a part of an ongoing program to optimize C-8 methoxy FQs against mycobacteria, we also have focused our attention on exploring the effect of increasing the lipophilic character at C-7 position of FQs by forming methylene- or ethylene-linked isatin-FQ conjugates. Isatin (1*H*-indole-2,3-dione) is an endogenous compound identified in many organisms [18], and its derivatives are reported to show variety of biological activity like antibacterial [19], antifungal [20], anti-HIV [21] and anti-TB activity [22], so great interests have been placed on these bioactive substances. In the present report, a series of novel BLFX ethylene isatin derivatives were designed and synthesized. Our primary objective was to optimize the potency of these compounds against mycobacteria.

2. Results and discussion

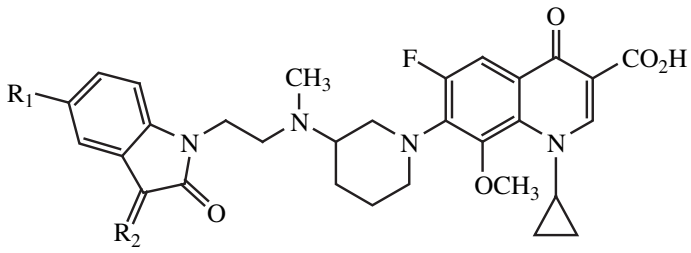
2.1. Chemistry

Detailed synthetic pathways to BLFX ethylene isatin derivatives **3a**–**n** are depicted in Scheme 1. Isatins **1a**,**b** were alkylated with 1,2-dibromoethane in the presence of anhydrous potassium carbonate to give N-(2-bromoethyl)isatins **2a**,**b** in a yield of 53–68% according to the reported procedures [20]. Nucleophilic substitution reactions of compounds **2a**,**b** with BLFX [1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylamino- piperidin-1-yl)-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid] were performed in the presence of anhydrous potassium carbonate in N,N-dimethylformamide at 40 °C to afford BLFX derivatives **3a**,**b** (57–59%). Subsequent condensations of compounds **3a**,**b** with requisite substituted amine hydrochlorides in the presence of sodium bicarbonate formed other derivatives (Schiff's bases) **3c**–**n** (63–85%).



Scheme 1. Synthesis of Balofloxacin derivatives 3a-n.

Table 1
Structures, lipophilicity, antimycobacterial activity and cytotoxicity of compounds **3a**–**n**.



3a-n

Compound	R_1	R_2	Clog *P* (log *P*)	MIC (μg/mL)				CC_{50}[c] (μM)
				M.p.[a]	M s[b]	MTB 09710	MTB H37Rv TCC 27294	
3a	H	O	1.10 (2.44)	25	25	–	–	136.9
3b	F	O	1.43 (2.60)	25	12.5	64	–	96.2
3c	H	NOH	0.79 (2.83)	12.5	12.5	–	–	148.2
3d	F	NOH	0.93 (2.99)	6.25	12.5	16	–	305.6
3e	H	$NOCH_3$	1.38 (3.10)	3.12	6.25	–	–	15.0
3f	F	$NOCH_3$	1.52 (3.25)	3.12	6.25	–	–	12.8
3g	H	$NOCH_2CH_3$	1.91 (3.43)[d]	3.12	6.25	8	–	60.5
3h	F	$NOCH_2CH_3$	2.05 (3.59)[d]	3.12	6.25	<0.5	0.25	53.1
3i	H	$NNHCSNH_2$	0.79 (2.36)[d]	3.12	6.25	8	–	293.8
3j	F	$NNHCSNH_2$	0.94 (2.52)[d]	3.12	6.25	4	8.0	312.9
3k	H	$NNHCONH_2$	0.13 (1.80)	12.5	12.5	–	–	56.1
3l	F	$NNHCONH_2$	0.27 (1.96)	6.25	6.25	32	–	57.0
3m	H	$NNHC_6H_5$	2.24 (4.08)	3.12	6.25	–	–	<6.1
3n	F	$NNHC_6H_5$	2.38 (4.24)	3.12	6.25	–	–	<6.0
BLFX			−0.27(1.74)[d]	0.78	0.39	16	8.0	317.0
MXFX			−0.08(1.68)	0.20	0.10	<0.5	0.25	160.8

[a] *M.p.*: *M. phlei* CMCC 93201.
[b] *M.s.*: *M. smegmatis* CMCC 93202.
[c] CC_{50}: The 50% cytotoxic concentration.
[d] The experimental log *P* values by HPLC of compounds **3g**–**j** and BLFX are 0.96, 1.29, 1.40, 1.80 and −1.11, respectively.

2.2. Lipophilicity

Lipophilicity of the target compounds **3a**–**n** and the parent compound BLFX and MXFX, is expressed in the term of their Clog *P* or log *P* values. As shown in Table 1, a remarkable improvement in the lipophilicity of the target compounds **3a**–**n**, as seen from the Clog *P* or log *P* values (0.13–2.38 or 1.80–4.24, statistically significant at $p < 0.001$ using *t* test), which were much more than that of BLFX (−0.27 or 1.74).

Compounds **3g**–**j** were chosen for further evaluation their experimental log *P* values by HPLC, and compared with BLFX (Table 1). These results indicated that the lipophilicity of derivatives **3g**–**j** (experimental log *P* values arrange from 0.96 to 1.80) was much more than BLFX (−1.11), which was consistent with lipophilicity profiles based on Clog *P* or log *P* values calculated by softwares. This may be rendering them more capable of penetrating various biomembrane, consequently improving their permeation properties toward mycobacterial cell membrane. In other words, the improvement of the lipophilic character of compounds **3a**–**n** probably enhances their antimycobacterial activity.

2.3. Pharmacology

The target compounds **3a**–**n** were initially evaluated for their *in vitro* antimycobacterial activity against *M. phlei* CMCC 93201 and *M. smegmatis* CMCC 93202 using serial double dilution technique in duplicate [16]. The minimum inhibitory concentration (MIC) is defined as the concentration of the compound required to give complete inhibition of mycobacterial growth, and MICs of the target compounds along with BLFX and MXFX for comparison are presented in Table 1. These data suggested that the target compounds **3a**–**n** showed considerable potency in inhibiting the growth of *M. phlei* CMCC 93201 and *M. smegmatis* CMCC 93202 (MIC: 3.12–25 μg/mL), although less active than that of the parent BLFX (MIC: 0.39–0.78 μg/mL).

Compounds **3b**, **3d**, **3g**–**j** and **3l**, characterized by their better activity, were chosen for further evaluation their *in vitro* activity against MTB 09710 clinical isolate, and then compounds **3h** and **3g** against MTB H37Rv ATCC 27294 using rapid direct susceptibility test technique [24,25]. The MICs of the seven derivatives, along with BLFX and MXFX for comparison, are presented in Table 1. Among them, compounds **3g**–**j** (MIC: <0.5–8 μg/mL) were more active than the parent BLFX (MIC: 16 μg/mL) against MTB 09710. In particular, compound **3h** (MIC: 0.25– < 0.5 μg/mL) was found to be comparable to MXFX, and ≥32 fold more potent than BLFX against MTB 09710 and MTB H37Rv ATCC 27294.

The target compounds **3a**–**n** were subsequently examined for toxicity (CC_{50}) in a mammalian Vero cell line [23]. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a formazan product and the results are reported in Table 1. Herein, the cytotoxicity of compounds **3e**, **3f**, **3m** and **3n** (CC_{50}: <6.0–15.0 μM) is much more than the other derivatives (CC_{50}: 53.1–312.9 μM).

2.4. Conclusion

In summary, a series of novel BLFX ethylene isatin derivatives **3a**–**n** were designed, synthesized and characterized by ^{1}H NMR, MS and HRMS. These derivatives were initially evaluated for their *in*

vitro antimycobacterial activity against *M. phlei* CMCC 93201 and *M. smegmatis* CMCC 93202. Compounds **3b**, **3d**, **3g**–**j** and **3l** were further evaluated their activity against MTB 09710 clinical isolate, and then compounds **3h** and **3g** against MTB H37Rv ATCC 27294. The preliminary evaluation results showed that all of the synthesized compounds with much more lipophilic than that of the parent BLFX, were less active than BLFX against *M. phlei* CMCC 93201 and *M. smegmatis* CMCC 93202. Compounds **3g**–**j** were more active than BLFX against MTB 09710, while the other three compounds (**3b**, **3d** and **3l**) were not. It was noted that the activity of compound **3h** was comparable to MXFX, and ≥32 fold more potent than BLFX against MTB 09710 and MTB H37Rv ATCC 27294. The above results demonstrated that the lipophilicity of the tested compounds was not the sole parameter affecting antimycobacterial activity. However, improvement in the lipophilicity of the compounds containing a 5-fluoroisatin moiety did increase the antimycobacterial activity, when compared with corresponding analogs containing an isatin moiety against MTB 09710, as seen from the MICs of compounds **3h** (<0.5 μg/mL) *versus* **3g** (8 μg/mL), or **3j** (4 μg/mL) *versus* **3i** (8 μg/mL).

Antimycobacterial activity of Schiff's bases (**3c**–**n**) of derivatives **3a**,**b** generally improved. The relative contribution of imines of the Schiff's bases to activity against MTB 09710 is as follows: ethyloxime > thiosemicarbazone > oxime > semicarbazone. The relative contribution of imines to cytotoxicity as follows: phenylhydrazone > methyloxime > semicarbazone ≈ ethyloxime > oxime > thiosemicarbazone.

3. Experimental section

3.1. Chemistry

Melting points were determined in open capillaries and uncorrected. Clog *P* was calculated by CLOGP module in sybyl 7.3 software. Log *P* values were calculated with Chem office 2009 software. HPLC was preformed using a Shimadzu LC-10Avp with SPD-10Avp UV detector (Shimadzu) and a Class VP 6.x workstation. The column used was a Diamonsil C18 5-μm 250 × 4.6 mm column (Dikma Technologies). ^{1}H NMR and ^{13}C NMR spectra were determined on a Varian Mercury-400 spectrometer in DMSO-d_6 or $CDCl_3$ using tetramethylsilane (TMS) as an internal standard. Electrospray ionization (ESI) mass spectra and high resolution mass spectra (HRMS) were obtained on a MDSSCIEX Q-Tap mass spectrometer and AccuTOF CS JMS-T100CS (JEOL) mass spectrometer, respectively. Fast Atom Bombardment (FAB) mass spectra and high resolution mass spectra (HRMS) were obtained on a MICROMASS AutoSpec Ultima-TOF mass spectrometer. Unless otherwise noted, the reagents were obtained from commercial supplier and were used without further purification. TLC was performed on silica gel plates (Merck, ART5554 60F_{254}).

3.2. Synthesis

3.2.1. N-(2-Bromoethyl)isatin (*2a*)

A suspension of isatin (**1a**, 4.41 g, 30 mmol), anhydrous potassium carbonate (12.51 g, 90 mmol) and 1,2-dibromoethane (16.83 g, 90 mmol) in N,N-dimethylformamide (100 mL) was stirred at room temperature for 24 h and filtered. The filtrate was concentrated under reduced pressure. The residue was poured into water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with saturated brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel) eluted with petroleum ether and ethyl acetate (*v*: *v* = 5: 1) to give the title compound **2a** (5.19 g, 68%) as anacaratsolid, mp: 126–128 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 3.62 (2H, t, *J* = 6.8, CH_2CH_2Br), 4.15 (2H, t, *J* = 6.8, CH_2CH_2Br), 7.00 (1H, d, *J* = 7.6), 7.15 (1H, t, *J* = 7.6), 7.60–7.65 (2H, m). ESI-MS: *m*/*z* 254 $(M + H)^+$, 256 $(M + 2 + H)^+$.

*3.2.1.1. N-(2-Bromoethyl)-5-fluoroisatin (**2b**).* The title compound was obtained in a similar manner as for the preparation of **2a** (53%) as anacaratsolid, mp: 109–110 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 3.62 (2H, t, *J* = 6.4, CH_2CH_2Br), 4.11 (2H, t, *J* = 6.4, CH_2CH_2Br), 6.98–7.00 (1H, m), 7.26–7.35 (2H, m). ESI-MS: *m*/*z* 272 $(M + H)^+$, 274 $(M+2 + H)^+$.

3.2.2. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-(2-isatinylethyl)] aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (*3a*)

A suspension of **2a** (5.08 g, 20 mmol), BLFX (7.78 g, 20 mmol), anhydrous potassium carbonate (8.34 g, 60 mmol) in N,N-dimethylformamide (200 mL) was stirred at 40 °C for 27.5 h and concentrated under reduced pressure. To the residue was added water (300 mL), stirred at room temperature for 0.5 h and then filtered. The solid obtained was dissolved in 4 N HCl (150 mL), and washed with methylene chloride (3 × 100 mL). The water layer was adjusted pH 7.0 with 10% NaOH solution and filtered. The crude product was purified by column chromatography (silica gel) eluted with methylene chloride and methanol (*v*: *v* = 20: 1) to give the title compound **3a** (6.65 g, 59%) as anacaratsolid, mp: 151–154 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.84–0.91 (2H, m, *cyclopropyl*-H), 0.97–1.02 (2H, m, *cyclopropyl*-H), 1.54–1.56 (1H, m), 1.64–1.67 (1H, m), 1.83–1.86 (1H, m), 2.16–2.20 (1H, m), 2.55 (3H, s, NCH_3), 3.03 (1H, t, *J* = 11.6), 3.16–3.18 (2H, m), 3.30 (1H, d, *J* = 11.6), 3.65 (1H, d, *J* = 11.6), 3.74 (3H, s, OCH_3), 3.92–3.95 (1H, m, *cyclopropyl*-H), 4.05 (2H, t, *J* = 5.2, CH_2 of linker), 4.36 (2H, t, *J* = 5.2, CH_2 of linker), 7.11–8.34 (6H, m, Ar-H), 8.98 (brs, 1H, D_2O exchangeable, COOH). ESI-MS: *m*/*z* 563 $(M + H)^+$. HRMS-ESI: *m*/*z* Calcd. for $C_{30}H_{32}FN_4O_6$ $(M + H)^+$: 563.23059; Found 563.22917.

*3.2.2.1. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(5-fluoroisatinyl)ethyl] aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3b**).* The title compound was obtained in a similar manner as for the preparation of **3a** (57%) as anacaratsolid, mp: 155–158 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.84–0.90 (2H, m, *cyclopropyl*-H), 0.97–1.01 (2H, m, *cyclopropyl*-H), 1.41–1.44 (1H, m), 1.63–1.66 (1H, m), 1.80–1.83 (1H, m), 2.50 (3H, s, NCH_3), 3.00–3.06 (3H, m), 3.30 (1H, d, *J* = 11.2), 3.60 (1H, d, *J* = 11.6), 3.74 (3H, s, OCH_3), 3.92–3.95 (1H, m, *cyclopropyl*-H), 4.05 (2H, t, *J* = 5.2, CH_2 of linker), 4.35 (2H, t, *J* = 5.2, CH_2 of linker), 7.39–8.35 (5H, m, Ar-H). ESI-MS: *m*/*z* 581 $(M + H)^+$. HRMS-ESI: *m*/*z* Calcd. for $C_{30}H_{31}F_2N_4O_6$ $(M + H)^+$: 581.22117; Found 581.22235.

3.2.3. General procedure for the preparation of compounds *3c*–*n*

The general procedure for preparing compounds **3c**–**n** was described as follows. To a solution of substituted amine hydrochlorides (3 mmol) and sodium bicarbonate (0.25 g, 3 mmol) dissolved in water (10 mL) was added dropwise a solution of **3a**, **b** (1 mmol) in methanol (10 mL) at room temperature over 5 min. The reaction mixture was stirred at the same tempareture for 12–24 h (monitored until the substrate disappeared by TLC, $v_{\text{methylene chloride}}$: v_{methanol} = 5: 1). After removal of the methanol under reduced pressure, the reaction mixture was diluted with water (20 mL) and stirred for 10 min, and then filtered. The solid crude product was purified by column chromatography (silica gel) eluted with methylene chloride and methanol (*v*: *v* = 20: 1) to give the title compounds **3c**–**n** (63–85%).

*3.2.3.1. 1-Cyclopropyl-6-fluoro-7-{3-[N-methyl-N-2-(isatinyl-β-hydroximino)ethyl] aminopiperidin-1-yl}-1,4-dihydro-8-methoxy–4-oxoquinoline-3-carboxylic acid (**3c**).* Yield: 85%. mp: 188–192 °C. ^{1}H NMR

(DMSO-d_6, 400 MHz) δ_H 0.83–0.91 (2H, m, *cyclopropyl*-H), 0.98–1.02 (2H, m, *cyclopropyl*-H), 1.48–1.51 (1H, m), 1.64–1.67 (1H, m), 1.82–1.85 (1H, m), 2.13–2.16 (1H, m), 2.54 (3H, s, NCH_3), 3.04 (1H, t, $J = 10.8$), 3.11–3.13 (2H, m), 3.28–3.31 (1H, m), 3.62 (1H, d, $J = 8.0$), 3.74 (3H, s, OCH_3), 3.91–3.96 (1H, m, *cyclopropyl*-H), 4.08 (2H, t, $J = 4.8$, CH_2 of linker), 4.37 (2H, t, $J = 4.8$, CH_2 of linker), 7.05–8.35 (6H, m, Ar-H). ESI-MS: *m/z* 578 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{30}H_{33}FN_5O_6$ $(M + H)^+$: 578.24149; Found 578.23997.

*3.2.3.2. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(5-fluorinisatinyl-β- hydroximino)ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3d**).* Yield: 82%. mp: 193–194 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.84–0.87 (2H, m, *cyclopropyl*-H), 1.00–1.01 (2H, m, *cyclopropyl*-H), 1.41–1.44 (1H, m), 1.62–1.65 (1H, m), 1.79–1.82 (1H, m), 2.09–2.12 (1H, m), 2.48 (3H, s, NCH_3), 3.00–3.03 (3H, m), 3.20 (1H, d, $J = 10.8$), 3.58 (1H, d, $J = 10.8$), 3.72 (3H, s, OCH_3), 3.92–3.95 (1H, m, *cyclopropyl*-H), 4.07 (2H, t, $J = 4.8$, CH_2 of linker), 4.35 (2H, t, $J = 4.8$, CH_2 of linker), 7.27–8.33 (5H, m, Ar-H). ESI-MS: *m/z* 596 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{30}H_{32}F_2N_5O_6$ $(M + H)^+$: 596.23206; Found 596.22956.

*3.2.3.3. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(β-methoxyiminoisatinyl) ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3e**).* Yield: 85%. mp: 156–158 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.84–0.91 (2H, m, *cyclopropyl*-H), 0.99–1.04 (2H, m, *cyclopropyl*-H), 1.54–1.56 (1H, m), 1.65–1.68 (1H, m), 1.83–1.87 (1H, m), 2.17–2.19 (1H, m), 2.58 (3H, s, NCH_3), 3.04 (1H, t, $J = 10.4$), 3.17 (2H, m), 3.28–3.32 (1H, m), 3.65 (1H, d, $J = 10.4$), 3.74 (3H, s, OCH_3), 3.92–3.96 (1H, m, *cyclopropyl*-H), 4.06 (2H, t, $J = 5.2$, CH_2 of linker), 4.20 (3H, s, $NOCH_3$), 4.36 (2H, t, $J = 5.2$, CH_2 of linker), 7.06–8.34 (6H, m, Ar-H), 8.99 (brs, 1H, D_2O exchangeable, COOH). ESI-MS: *m/z* 592 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{31}H_{35}FN_5O_6$ $(M + H)^+$: 592.25714; Found 592.26033.

*3.2.3.4. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(5-fluorinisatinyl-β- methoxyimino)ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3f**).* Yield: 79%. mp: 169–171 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.85–0.91 (2H, m, *cyclopropyl*-H), 0.99–1.03 (2H, m, *cyclopropyl*-H), 1.54–1.67 (2H, m), 1.82–1.86 (1H, m), 2.17–2.20 (1H, m), 2.55 (3H, s, NCH_3), 3.03 (1H, t, $J = 10.4$), 3.16–3.19 (2H, m), 3.28–3.31 (1H, m), 3.64–3.66 (1H, m), 3.75 (3H, s, OCH_3), 3.93–3.97 (1H, m, *cyclopropyl*-H), 4.07 (2H, t, $J = 4.8$, CH_2 of linker), 4.21 (3H, s, $NOCH_3$), 4.36 (2H, t, $J = 4.8$, CH_2 of linker), 7.28–8.32 (5H, m, Ar-H), 9.04 (brs, 1H, D_2O exchangeable, COO*H*). ESI-MS: *m/z* 610 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{31}H_{34}F_2N_5O_6$ $(M + H)^+$: 610.24771; Found 610.24786.

*3.2.3.5. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(β-ethoxyiminoisatinyl) ethyl]aminopiperidin-1-yl]-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3g**).* Yield: 79%. mp: 101–103 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 0.82–0.88 (2H, m, *cyclopropyl*-H), 1.07–1.11 (2H, m, *cyclopropyl*-H), 1.41–1.45 (4H, m, $NOCH_2CH_3$, *piperidinyl*-H), 1.71–1.74 (1H, m), 1.82–1.84 (1H, m), 2.06–2.10 (1H, m), 2.52 (3H, s, NCH_3), 2.79–2.83 (1H, m), 2.99 (1H, t, $J = 10.8$), 3.12 (1H, t, $J = 10.8$), 3.36 (1H, d, $J = 12.4$), 3.62 (1H, d, $J = 12.4$), 3.73 (3H, s, OCH_3), 3.84–3.90 (1H, m, *cyclopropyl*-H), 4.13 (2H, t, $J = 5.6$, CH_2 of linker), 4.48–4.56 (4H, m, CH_2 of linker, $NOCH_2CH_3$), 7.00–8.43 (6H, m, Ar-H). ESI-MS: *m/z* 606 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{32}H_{37}FN_5O_6$ $(M + H)^+$: 606.27279; Found 606.27614.

*3.2.3.6. 1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-{3-[N-methyl-N-2-(5- fluorinisatinyl-β-ethoxyimino)ethyl]aminopiperidin-1-yl}-4-oxoquinoline-3-carboxylic acid (**3h**).* Yield: 71%. mp: 107–110 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 0.87–0.91 (2H, m, *cyclopropyl*-H), 1.11–1.14 (2H, m, *cyclopropyl*-H), 1.41–1.46 (4H, m, $NOCH_2CH_3$, *piperidinyl*-H), 1.72–1.76 (1H, m), 1.82–1.85 (1H, m), 2.08 (1H, t, $J = 12.0$), 2.53 (3H, s, NCH_3), 2.80 (1H, s), 2.98 (1H, t, $J = 11.2$), 3.13 (1H, t, $J = 11.2$), 3.37 (1H, d, $J = 12.0$), 3.62 (1H, d, $J = 12.0$), 3.72 (3H, s, OCH_3), 3.86–3.90 (1H, m, *cyclopropyl*-H), 4.13 (2H, t, $J = 5.2$, CH_2 of linker), 4.48–4.58 (4H, m, CH_2 of linker, $NOCH_2CH_3$), 7.05–8.44 (5H, m, Ar-H). ^{13}C NMR ($CDCl_3$, 400 MHz) δ_C: 9.16, 9.34, 14.61, 24.63, 29.82, 32.99, 38.87, 39.60, 51.30, 55.86, 56.14, 61.10, 62.41, 72.91, 108.39, 108.59, 109.16, 115.73, 122.88, 127.69, 132.42, 132.75, 138.59, 143.25, 143.41, 145.47, 150.62, 154.73, 157.20, 163.75, 163.99, 172.93. ESI-MS: *m/z* 624 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{32}H_{36}F_2N_5O_6$ $(M + H)^+$: 624.26336; Found 624.26651.

*3.2.3.7. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(β-thiosemicarbazo isatinyl)ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3i**).* Yield: 80%. mp: 174–176 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.85–0.89 (2H, m, *cyclopropyl*-H), 1.05–1.07 (2H, m, *cyclopropyl*-H), 1.22–1.25 (1H, m), 1.60–1.63 (1H, m), 1.74–1.77 (1H, m), 1.89–2.00 (1H, m), 2.35 (3H, s, NCH_3), 2.67–2.72 (1H, m), 2.80–2.85 (1H, m), 3.00–3.06 (1H, m), 3.29 (1H, d, $J = 10.0$), 3.52 (1H, d, $J = 10.0$), 3.72 (3H, s, OCH_3), 3.97–4.00 (1H, m, *cyclopropyl*-H), 4.13 (2H, t, $J = 4.8$, CH_2 of linker), 4.37 (2H, t, $J = 4.8$, CH_2 of linker), 7.13–8.40 (6H, m, Ar-H), 8.73 (brs, 1H, D_2O exchangeable, $NNHCSNH_2$), 9.06 (brs, 1H, D_2O exchangeable, COOH). HRMS-FAB: *m/z* Calcd. for $C_{31}H_{35}FN_7O_5S$ $(M + H)^+$: 636.2404; Found 636.2355.

*3.2.3.8. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(5-fluorinisatinyl-β- thiosemicarbazo)ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3j**).* Yield: 70%. mp: 191–193 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.86–0.90 (2H, m, *cyclopropyl*-H), 0.98–1.09 (2H, m, *cyclopropyl*-H), 1.25–1.34 (1H, m), 1.61–1.64 (1H, m), 1.75–1.78 (1H, m), 2.02–2.05 (1H, m), 2.40 (3H, s, NCH_3), 2.75–2.79 (1H, m), 2.90 (1H, t, $J = 11.2$), 3.02 (1H, t, $J = 11.2$), 3.29 (1H, d, $J = 12.0$), 3.54 (1H, d, $J = 9.6$), 3.72 (3H, s, OCH_3), 3.95–4.00 (1H, m, *cyclopropyl*-H), 4.12 (2H, t, $J = 4.8$, CH_2 of linker), 4.36 (2H, t, $J = 4.8$, CH_2 of linker), 7.24–8.38 (5H, m, Ar-H), 8.81 (brs, 1H, D_2O exchangeable, $NNHCSNH_2$), 9.14 (brs, 1H, D_2O exchangeable, COO*H*). ESI-MS: *m/z* 654 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{31}H_{34}F_2N_7O_5S$ $(M + H)^+$: 654.23102; Found 654.23211.

*3.2.3.9. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(β-semicarbazoisatinyl) ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3k**).* Yield: 73%. mp: 199–201 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.84–0.90 (2H, m, *cyclopropyl*-H), 0.99–1.06 (2H, m, *cyclopropyl*-H), 1.53–1.55 (1H, m), 1.65–1.68 (1H, m), 1.83–1.86 (1H, m), 2.16–2.18 (1H, m), 2.72 (3H, s, NCH_3), 3.04 (1H, t, $J = 10.4$), 3.13–3.23 (2H, m), 3.28–3.31 (1H, m), 3.64 (1H, d, $J = 10.0$), 3.74 (3H, s, OCH_3), 3.92–3.96 (1H, m, *cyclopropyl*-H), 4.10 (2H, t, $J = 5.0$, CH_2 of linker), 4.37 (2H, t, $J = 5.0$, CH_2 of linker), 6.09 (brs, 2H, D_2O exchangeable, $NNHCONH_2$), 7.07–8.36 (6H, m, Ar-H), 8.89 (brs, 1H, D_2O exchangeable, $NNHCONH_2$), 10.33 (brs, 1H, D_2O exchangeable, COOH). ESI-MS: *m/z* 620 $(M + H)^+$. HRMS-FAB: *m/z* Calcd. for $C_{31}H_{35}FN_7O_6$ $(M + H)^+$: 620.2633; Found 620.2667.

*3.2.3.10. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(5-fluorinisatinyl-β- semicarbazo)ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3l**).* Yield: 68%. mp: 197–199 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.83–0.89 (2H, m, *cyclopropyl*-H), 0.99–1.05 (2H, m, *cyclopropyl*-H), 1.45–1.47 (1H, m), 1.63–1.66 (1H, m), 1.80–1.83 (1H, m), 2.11–2.14 (1H, m), 2.49 (3H, s, NCH_3), 3.01–3.16 (3H, m), 3.27 (1H, d, $J = 12.0$), 3.60 (1H, d, $J = 8.4$), 3.74 (3H, s, OCH_3), 3.92–3.96 (1H, m, *cyclopropyl*-H), 4.08–4.13 (2H, m, CH_2 of linker), 4.35–4.38 (2H, m, CH_2 of linker), 6.94 (brs, 2H, D_2O exchangeable, $NNHCONH_2$), 7.21–8.34 (5H, m, Ar-H), 8.80 (brs, 1H, D_2O exchangeable, $NNHCONH_2$). ESI-MS: *m/z* 638 $(M + H)^+$.

HRMS-ESI: *m/z* Calcd. for $C_{31}H_{34}F_2N_7O_6$ $(M + H)^+$: 638.25386; Found 638.24956.

*3.2.3.11. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(β-phenylhydrazo isatinyl)ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3m**).* Yield: 78%. mp: 151–153 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.80–0.84 (2H, m, *cyclopropyl*-H), 0.93–1.00 (2H, m, *cyclopropyl*-H), 1.16–1.22 (1H, m), 1.34–1.37 (1H, m), 1.61–1.63 (1H, m), 1.77–1.80 (1H, m), 2.05–2.08 (1H, m), 2.49 (3H, s, NCH_3), 2.89–3.03 (3H, m), 3.54 (1H, d, $J = 10.0$), 3.69 (3H, s, OCH_3), 3.86–3.89 (1H, m, *cyclopropyl*-H), 4.17 (2H, t, $J = 5.0$, CH_2 of linker), 4.44 (2H, t, $J = 5.0$, CH_2 of linker), 7.03–8.66 (11H, m, Ar-H), 12.69 (brs, 1H, D_2O exchangeable, COOH). ESI-MS: *m/z* 653 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{36}H_{38}FN_6O_5$ $(M + H)^+$: 653.28877; Found 653.28580.

*3.2.3.12. 1-Cyclopropyl-6-fluoro-8-methoxy-7-{3-[N-methyl-N-2-(5-fluorinisatinyl-β- phenylhydrazo)ethyl]aminopiperidin-1-yl}-1,4-dihydro-4-oxoquinoline-3-carboxylic acid (**3n**).* Yield: 63%. mp: 166–167 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 0.82–0.90 (2H, m, *cyclopropyl*-H), 0.93–1.00 (2H, m, *cyclopropyl*-H), 1.16–1.22 (1H, m), 1.38–1.40 (1H, m), 1.62–1.64 (1H, m), 1.78–1.81 (1H, m), 2.07–2.10 (1H, m), 2.49 (3H, s, NCH_3), 2.99–3.04 (3H, m), 3.55 (1H, d, $J = 7.2$), 3.66 (3H, s, OCH_3), 3.86–3.88 (1H, m, *cyclopropyl*-H), 4.17 (2H, t, $J = 4.8$, CH_2 of linker), 4.43 (2H, t, $J = 4.8$, CH_2 of linker), 7.03–8.33 (10H, m, Ar-H), 12.69 (brs, 1H, D_2O exchangeable, COOH). ESI-MS: *m/z* 671 $(M + H)^+$. HRMS-ESI: *m/z* Calcd. for $C_{36}H_{37}F_2N_6O_5$ $(M + H)^+$: 671.27935; Found 671.28140.

3.3. MIC determination

The target compounds **3a**–**n** were screened for their *in vitro* activity. The compounds were dissolved in DMSO at concentrations from 200 to 0.1 μg/ml. Two-fold dilutions of compounds and the tested strains were prepared in 54 medium in a volume of 150 μL in 96-well microplates. The plates were incubated at 37 °C for 72 h.

Compounds **3b**, **3d**, **3g**–**j** and **3l** were chosen for further evaluation their *in vitro* activity against MTB 09710 clinical isolate, and then compounds **3h** and **3g** against MTB H37Rv ATCC 27294 using rapid direct susceptibility test technique [24,25]. The tested compounds were dissolved in distilled water and two-fold diluted at concentrations from 128 to 0.5 μg/ml (for MTB 09710) or 32 to 0.125 μg/ml (for MTB H37Rv ATCC 27294). The two strains were obtained from Jiangsu Province Hospital, Nanjing, China.

3.4. Cytotoxicity

The target compounds **3a**–**n** were further examined for toxicity (CC_{50}) in a mammalian Vero cell line at concentrations from 1000 to 4 μg/ml. The Vero cells were maintained in culture medium (Minimum Essential Medium with Earle's salt, supplemented with 10% fetal bovine serum) at 37 °C under 5% CO_2. Cells were seeded in 96-well plates at the plating density of 1×10^4 cells per well and allowed to recover for 24 h. Culture medium was replaced by assay medium containing the compound to be tested or drug-free. After 72 h of exposure, cells were harvested and cell viability was assessed by MTT assay. The CC_{50} values were calculated by Bliss analyses.

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