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Synthesis and antimycobacterial evaluation of various 7-substituted ciprofloxacin derivatives

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Abstract—Tuberculosis continues to be a major cause of morbidity and mortality all over the world. Various 7-substituted ciprofloxacin derivatives were synthesized and evaluated for antimycobacterial activity in vitro and in vivo against *Mycobacterium tuberculosis* and for inhibition of the supercoiling activity of DNA gyrase from *Mycobacterium smegmatis*. Preliminary results indicated that most of the compounds demonstrated better in vitro antimycobacterial activity against *M. tuberculosis* than ciprofloxacin. Compound 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[[N^4-[1′-(5-methylisatinyl-β-semicarbazo)]methyl]N^1-piperazinyl]-3-quinoline carboxylic acid (**3h**) decreased the bacterial load in spleen tissue with 0.76-$\log_{10}$ protections and was considered to be moderately active in reducing bacterial count in spleen. The results demonstrated the potential and importance of developing new quinolone derivatives against mycobacterial infections.

1. Introduction

Tuberculosis (TB) is one of the most common infectious diseases known to humans. About 32% of the world's population (1.9 billion people) is infected with TB. Every year, approximately 8 million of the infected people develop active TB, and almost 2 million of these infected people die from the disease,[1] a life lost to TB every 15 s. The incidence of TB infection has steadily risen in the last decade, and this increase can be attributed to a similar increase in human immunodeficiency virus (HIV) infection.[2] The association of TB and HIV infections is so dramatic that, in some cases, nearly two-thirds of the patients diagnosed with TB are also HIV-1 seropositive.[3] Furthermore, numerous studies have shown that TB is a cofactor in the progression of HIV infection.[4] The reemergence of TB infection is further complicated by an increase in cases that are resistant to conventional antitubercular drug therapy. As a consequence of HIV epidemic, and the lack of potency of multidrug regimens, drugresistance has become more evident and the development of novel mechanism-based antitubercular agents has become a high priority area globally.[5,6] Fluoroquinolones are used for the clinical control of multidrug resistant TB, that is, TB due to bacilli that are resistant to both isoniazid and rifampin. Several of the quinolone antibacterials, such as gatifloxacin, moxifloxacin, and sitafloxacin, have been examined as inhibitors of *Mycobacterium tuberculosis* (MTB), as well as other mycobacterial infections.[7] Quinolones inhibit bacterial type II topoisomerase, deoxyribonucleic acid (DNA) gyrase, and topoisomerase IV,[8] which are essential enzymes that maintain the supercoils in DNA. The incidence of mycobacterial resistance to fluoroquinolones is relatively low at the present time, and there are no reports of cross-resistance or antagonism with other classes of antimycobacterial agents.[9] One major factor relevant to the design of new antitubercular agents is the transport of compounds through the cell wall of mycobacteria. This is difficult since it is well known that mycolic acids and surface-associated lipids of these organisms form a transport barrier when compared to the cell wall of other eubacteria.[10] As a part of the study attempting to further optimize the quinolone antibacterials against MTB, we have explored the effect of increasing the lipophilic character at 7-position of ciprofloxacin on activity against MTB. In the present report, we describe the synthesis, antimycobacterial

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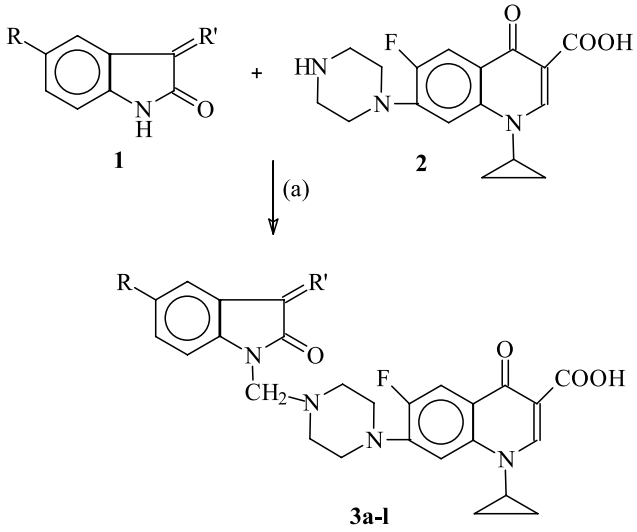
evaluation in vitro and in vivo against MTB and the tests of the ability of representative compounds to inhibit the supercoiling activity of *M. smegmatis* DNA gyrase.

2. Results and discussion

2.1. Synthesis

The general procedures for the preparation[11] of target compounds **3a–l** (Table 1) are described in Scheme 1. Isatin and its derivatives (**1**) react with formaldehyde and secondary amino (piperazino) function of ciprofloxacin (**2**) to form the required Mannich bases of ciprofloxacin in 68–89% yield. The purity of the synthesized compounds was checked by thin-layer chromatography, and the elemental analyses and the structures were identified by spectral data. In general, (IR) infrared spectra showed C=N (azomethine) peak at 1640/cm[1] and CH_2 (Mannich methylene) peak at 2860 and 2840/cm[1]. In the nuclear magnetic resonance spectra (^{1}H NMR), the signals of the respective protons of the prepared ciprofloxacin derivatives were verified on the basis of their chemical shifts, multiplicities, and coupling constants. The spectra showed a singlet at δ 4.8–5.1 ppm corresponding to –NCH_2N– group; multiplet at δ 3.8–4.1 ppm for piperazine proton; multiplet at δ 0.88–1.12 ppm for cyclopropyl proton and singlet at δ 8.6 ppm for C_2-H. The elemental analysis results were within ±0.4% of the theoretical values.



R = H, Cl, Br, CH_3
R = O, -NNHCONH$_2$, -NNHCSNH$_2$

(a) HCHO, C_2H_5OH, reflux, 24h

Scheme 1. Synthetic protocol of the titled compounds.

2.2. Antimycobacterial activity

All compounds were initially screened for their antimycobacterial activity at 6.25 μg/mL against MTB $H_{37}Rv$ strain by the Tuberculosis Antimicrobial Acquisition & Coordinating Facility (TAACF) in BACTEC 12B medium using the microplate Alamar Blue assay[12] (Table 1). Compounds exhibiting >90% inhibition in the initial screen were retested at and below 6.25 μg/mL using 2-fold dilution to determine the MIC. In the preliminary screening, all the compounds inhibited MTB with 95–100%. In the secondary level, five compounds (**3a**, **g–i**, and **k**) showed most promising activity with MIC of <2 nM and all the compounds were more potent than parent compound ciprofloxacin (MIC of 6.04 nM) except **3l**. When compared to moxifloxacin, compounds **3a**, **g–i** and **k** were more potent. Compound **3k** was found to be the most active compound with MIC of 1.21 nM and was five times more potent than ciprofloxacin in vitro. The preliminary antimycobacterial evaluation results show that compounds with bromo substitution in the C-5 position of isatinimino derivatives have shown promising results, and extensive struc-

Table 1. Physical and antimycobacterial properties of compounds **3a–l**

Compound	R	R′	Melting point (°C)	Yield (%)	Log *P*[a]	Antimycobacterial screening		Cytotoxicity	Selectivity index (IC_{50}/MIC)
						% Inhibition at 6.25μg/mL	MIC (nM)	IC_{50} Vero cells (nM)	
3a	H	O	230	63	1.01	99	1.59	>127.42	>80.13
3b	Cl	O	226	67	0.99	100	2.97	>19.05	>6.41
3c	Br	O	246	68	0.91	100	2.74	>17.56	>6.41
3d	CH_3	O	202	72	0.99	99	3.09	>19.82	>6.41
3e	H	–NNHCONH$_2$	235	79	1.92	100	2.85	>18.26	>6.41
3f	Cl	–NNHCONH$_2$	212	81	1.40	100	2.68	>17.18	>6.41
3g	Br	–NNHCONH$_2$	198	77	2.04	99	1.24	>99.77	>80.45
3h	CH_3	–NNHCONH$_2$	210	83	1.97	100	1.39	>111.29	>80.06
3i	H	–NNHCSNH$_2$	240	89	1.51	100	1.38	>110.89	>80.35
3j	Cl	–NNHCSNH$_2$	256	83	0.89	95	2.61	>16.72	>6.41
3k	Br	–NNHCSNH$_2$	208	86	1.66	100	1.21	>97.27	>80.38
3l	CH_3	–NNHCSNH$_2$	268	92	1.61	100	10.82	>10.82	>1.00
Ciprofloxacin	—	—	—	—	0.01	98	6.04	>30.21	>5.00
Moxifloxacin	—	—	—	—	−0.03	100	1.94	>15.58	>8.00

[a] The log *P* was calculated using online calculator (www.logp.com).

ture–activity correlation could be derived in the future with various other modifications.

The lipophilicity of the fluoroquinolones is well known to play an important role in the penetration of these compounds into bacterial cells.[13] Assuming that the issue of penetration is even more crucial for quinolone activity against mycobacteria,[14] our results demonstrated that simply increasing the lipophilic character at C-7 increased the activity, as seen from the log *P* values of the synthesized compounds (0.89–2.04), which were much more than the parent compound (0.01).

All the compounds were further examined for toxicity (IC_{50}) in a mammalian cell line, Vero cells by the TAACF. The compounds, which showed MIC of <2 nM, were nontoxic till 100 nM, and their selectivity indexes were more than 100. Vero cells are the cell lines used in the standard protocol followed in TAACF and they are developed from African monkey nephrocytes especially used for assessing the cytotoxicity.

2.3. Animal testing

Prior to animal screening, the maximum tolerated dose (MTD) was performed for a representative compound **3h** using C57BL/6 female mice by administration of a one-time dose/animal of an escalating dose of drug (100, 300, 500, and 1000 mg/kg). The nine mice (3 mice/dose) in each study were observed for a total of 1 week. Surviving mice were killed and organs examined for signs of overt toxicity. Compound **3h** showed no effect or adverse reactions/toxicity at the maximum dose.

Subsequently, compound **3h** was tested for efficacy against MTB at a dose of 300 mg/kg (Table 2) in 8- to 10-week-old female specific-pathogen-free C57BL/6-Ifngtm1ts (GKO) mice (Jackson Laboratories, Bar Harbor, ME, USA) exposed to a low-dose aerosol infection with MTB in a Glas-Col inhalation exposure system.[15] The virulent MTB strain Erdman (TMCC 107) has been used as the standard strain for drug testing. One day to postinfection, three mice were killed to verify bacterial uptake of 50–100 colony-forming unit (CFU)/mouse. Every treatment group consisted of five mice for every following time point. Treatment was started 18 days after infection and lasted up to 28 days postinfection. One control group of infected mice was killed at the start of treatment. A second group of infected but untreated mice was killed after the cessation of treatment. In this model, drug treatment began 20 days after inoculation of MTB into the animal. Bacterial counts were measured on day 28 in two tissues, viz., lungs and spleen, and compared with the counts from negative (untreated) controls (mean CFU in lung was 8.78 and in spleen was 6.84). Compound **3h** decreased the bacterial load in spleen tissue (mean CFU of 6.08) with 0.76-$\log_{10}$ protections and was considered to be moderately active in reducing bacterial count in spleen. In the lung tissue, the compound **3h** was found to be inactive (mean CFU of 9.26).

Table 2. In vivo activity data of **3h** and isoniazid against *Mycobacterium tuberculosis* in mice

Compound	Lungs (log CFU ± SEM)	Spleen (log CFU ± SEM)
Control	8.78 ± 0.12	6.84 ± 0.21
3h (300 mg/kg)	9.26 ± 0.19	6.08 ± 0.11
Isoniazid (25 mg/kg)	5.80 ± 0.18	3.14 ± 0.12

2.4. DNA gyrase inhibition

Since the compounds were fluoroquinolone derivatives, it was important to test whether they inhibit the enzyme DNA gyrase even after the modification. The ability of the compounds **3g**, **h**, and **k** to inhibit DNA supercoiling was tested using DNA gyrase from *M. smegmatis*. The gyrase subunits from *M. smegmatis* are >90% similar (Gyr A, 93.7%; Gyr B, 92%) to those present in MTB at the amino acid level.[16] Moxifloxacin was used as positive control in these assays, as it has been shown to be a potent inhibitor of DNA supercoiling of mycobacterial DNA gyrase.[17] Each of the new compounds tested showed dose-dependent inhibition. The representative data after carrying out experiments three times are shown in Figure 1A and B. From the data, it is clear that

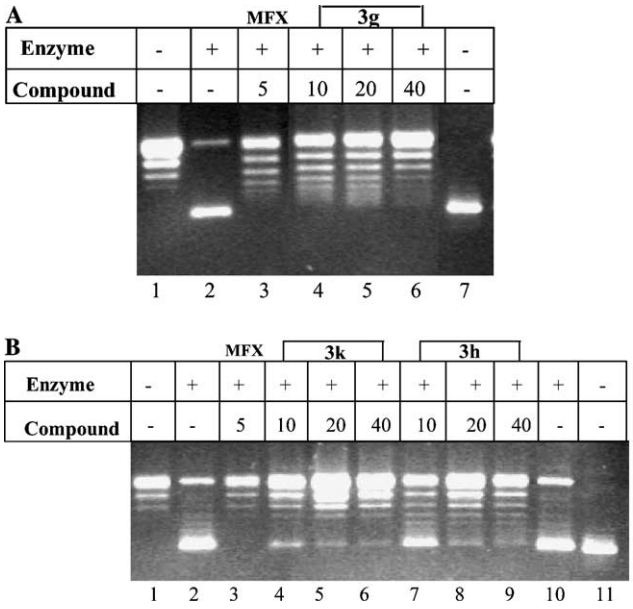


Figure 1. (A) DNA gyrase supercoiling assays to evaluate enzyme inhibition. The assays were performed as described in the Section 4. The enzyme was incubated with indicated concentrations of the compounds prior to the addition the reaction mixture. Moxifloxacin (5 μg/ml) was used as positive control for inhibition and 5% DMSO was used as solvent control. The samples after processing were analyzed on 0.8% agarose gels as described in the Section 4. Effect of compound **3g** on DNA gyrase. Lane 1, relaxed DNA substrate alone; lane 2, supercoiling reaction in the absence of any drug; lane 3, 5 μg/ml of moxifloxacin; lanes 4–6 have 10, 20, and 40 μg/ml of **3g**; lane 7 supercoiled plasmid DNA. (B) Effect of compounds **3k** and **h** on supercoiling activity. Lanes 1 and 11 have relaxed and supercoiled DNA, respectively. Lane 2, complete supercoiling reaction in the absence of any drug; lanes 3 and 10 are drug control and solvent (5% DMSO) controls, respectively. Lanes 4–6 and 7–9 have 10, 20, and 40 μg/ml of compounds **3k** and **h**, respectively.

the substituted compounds inhibit the DNA gyrase activity. However, none of the new compounds are as effective as moxifloxacin very likely due to the presence of different chemical groups. All the compounds used for the assays show IC_{50} values around 10 μg/mL. The results demonstrate that lipophilic quinolones retain their inhibitory property on DNA gyrase from mycobacteria.

3. Conclusion

This study has revealed that increasing the lipophilic side chain at C-7 had improved the antimycobacterial activity in vitro and had shown no cytotoxicity at the active concentration. The investigation on further structure–activity relationships and emergence of drug resistance are now in progress. Appropriate modification of other quinolones such as moxifloxacin, sparfloxacin, and sitafloxacin, which are more effective than ciprofloxacin, is likely to provide more effective inhibitors of the enzyme with improved efficacy.

4. Experimental

Melting points were taken on an electrothermal melting point apparatus (Buchi BM530) in open capillary tubes and are uncorrected. IR spectra (KBr disc) were run on Jasco IR Report 100 spectrometer. ^{1}H NMR spectra were scanned on a JEOL Fx 300 MHz NMR spectrometer using DMSO-d_6 as solvent. Chemical shifts are expressed in δ (ppm) relative to tertamethylsilane. Elemental analyses (C, H, and N) were performed on Perkin Elmer model 240C analyzer, and the data were within ± 0.4% of the theoretical values.

4.1. Synthesis of Compounds 3a–l

The general procedure for preparing **3a**–**l** was as follows. To a solution of 1-cyclopropyl-6-fluoro-1,4-dihydro-7-piperazin-1-yl-4-oxo quinoline-3-carboxylic acid (ciprofloxacin, 0.02 mol) in ethanol (50 mL), isatin and its derivatives (0.02 mol) and 37% formalin (1 mL) were added. The reaction mixture was heated under reflux for 24 h. On cooling, the precipitate was collected, washed with cold ethanol, and recrystallized from a mixture of DMF and water to give **3a**–**l** with 68–92% yield.

4.1.1. 1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[N^4-(isatinyl)methyl]-N^1-piperazinyl-3-quinoline carboxylic acid (3a). Yield: 63.2 %; mp: 230 °C; IR (KBr): 3010, 1736, 1720,1620, 1506, 1236, 1125 cm; ^{1}H NMR (DMSO-d_6) δ (ppm): 1.05–1.2 (m, 4H, cyclopropyl-H), 3.7–4.1 (m, 9H, -piperazine-H and cyclopropyl-H), 5.1 (s, 2H, –NCH_2N), 6.58–8.48 (m, 6H, Ar-H), 8.62 (s, 1H, C_2-H), 14.88 (br s, 1H, COOH); Calcd for $C_{26}H_{23}N_4O_5F$: C, 63.67; H, 4.73; N, 11.42. Found: C, 63.49; H, 4.71; N, 11.33.

4.1.2. 1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[[N^4-[1′-(5-methylisatinyl-β-semicarbazo)]methyl]-N^1-piperazinyl]-3-quinoline carboxylic acid (3h). Yield: 82.9%; mp: 210 °C; IR (KBr): 3010, 2850, 2840, 1736, 1645, 1620, 1506, 1236, 1125 cm; ^{1}H NMR (DMSO-d_6) δ (ppm): 0.88–1.12 (m, 4H, cyclopropyl-H), 1.82 (s, 3H, CH_3), 3.74–4.26 (m, 9H, -piperazine-H and cyclopropyl-H), 5.12 (s, 2H, –NCH_2N), 6.50–8.46 (m, 5H, Ar-H), 8.62 (s, 1H, C_2-H), 10.02 (s, 2H, $CONH_2$, D_2O exchangeable), 10.82 (s, 1H, NH, D_2O exchangeable), 14.86 (br s, 1H, COOH); Calcd for $C_{28}H_{28}N_7O_5F$: C, 59.89; H, 5.03; N, 17.46. Found: C, 60.01; H, 4.99; N, 17.43.

4.1.3. 1-Cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-[[N^4-[1′-(5-bromoisatinyl-β-thiosemicarbazo)]methyl]N^1-piperazinyl]-3-quinoline carboxylic acid (3k). Yield: 86.4%; mp: 268 °C; IR (KBr): 3010, 2852, 2840, 1735, 1640, 1620, 1506, 1236, 1125 cm; ^{1}H NMR (DMSO-d_6) δ (ppm): 0.88–1.10 (m, 4H, cyclopropyl-H), 3.74–4.26 (m, 9H, -piperazine-H and cyclopropyl-H), 5.16 (s, 2H, –NCH_2N), 6.62–8.40 (m, 5H, Ar-H), 8.62 (s, 1H, C_2-H), 10.62 (s, 2H, $CSNH_2$, D_2O exchangeable), 10.88 (s, 1H, NH, D_2O exchangeable), 14.86 (br s, 1H, COOH); Calcd for $C_{27}H_{25}N_7O_4SFBr$: C, 50.47; H, 3.92; N, 15.26. Found: C, 50.41; H, 3.99; N, 15.27.

4.2. Antimycobacterial activity

Antimicrobial susceptibility testing was performed using alamar blue susceptibility test (MABA) as reported earlier.[12] The lowest drug concentration effecting an inhibition of $\geqslant$90% was considered the MIC.

4.3. Cytoxicity assay

Cytotoxicity was assessed against Vero cells (CCL-81, American Type Culture Collection) following the earlier reported protocol.[12]

4.4. Maximum tolerated dose assay

Three healthy mice were given orally one single dose of the compound and were observed at regular times for any adverse effects. Four different concentrations were tested: 100, 300, 500, and 1000 mg/kg. The last dose was 2–5 times the dose used for efficacy testing of the compound in mice. After 7 days of observation the mice were sacrificed and the organs were studied by gross necropsy. In the case of abnormalities, the organs were fixed in formalin and further analyzed by extensive pathology analysis.

4.5. In vivo antimycobacterial activity

The animal testing was carried out as per the reported procedure.[15] The quinolones were suspended in 5% ethanol for treatment, and isoniazid (INH) was dissolved in distilled water. All compounds were administered by oral gavage in eight treatments for 5 days/week. After completion of therapy, the mice were killed by CO_2 inhalation. Spleens and left lungs were aseptically removed and disrupted in a tissue homogenizer. The number of viable organisms was determined by serial dilution of the homogenates on nutrient Middlebrook 7H11 agar plates (GIBCO BRL, Gaithersburg, MD, USA). The plates were incubated at 37 °C in ambient

air for 4 weeks prior to the counting of viable *M. tuberculosis* colonies (CFU). The viable counts were converted to logarithms, which were then evaluated by multiple-comparison analysis of variance by a one-way Dunnett test. Differences were considered significant at the 95% level of confidence.

4.6. DNA gyrase supercoiling assays

DNA gyrase was purified from *M. smegmatis* cells, and supercoiling assays were carried out as described previously.[17] For assessing the inhibition, the compounds **3g**, **k**, and **h** were dissolved in DMSO and incubated in the supercoiling assay mixture along with the enzyme. Control lanes (absence of compounds) contained DMSO (5%). The reaction samples were loaded on to 0.8% agarose gels and electrophoresed in TBE buffer after 12 h at room temperature. The gel is stained with ethidium bromide to visualize DNA topoisomers.

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