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Full Paper

Evaluation of antimycobacterial and DNA gyrase inhibition of fluoroquinolone derivatives

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The antimycobacterial activity (both in vitro and in vivo) and DNA gyrase inhibition of newly synthesized fluoroquinolone derivatives were tested against *Mycobacterium tuberculosis* H_{37}Rv and *Mycobacterium smegmatis*, respectively. Among the synthesized compounds, compound F11 was found to exhibit the most potent in vitro antimycobacterial activity with a MIC value of 0.78 μg/ml, and a selectivity index of more than 80 while not being cytotoxic to the Vero cell line up to 62.5 μg/ml. When evaluated for in vivo antimycobacterial activity, compound F11 demonstrated a paramount decrease of bacterial load in lung and spleen tissues compared to the control and better than the standard drug ciprofloxacin.

Key Words——DNA gyrase inhibition; fluoroquinolone derivatives; isatin derivatives; *Mycobacterium smegmatis; Mycobacterium tuberculosis*

Introduction

Infection with *Mycobacterium tuberculosis* remains a major global health emergency, which continues to overwhelm the population of the developing world, infecting two billion people or one-third of the world's population. It is second only to AIDS among other infectious diseases in causing deaths worldwide (Khasnobis et al., 2002). The search for novel anti-TB agents continues as a result of the ever increasing number of TB cases together with the advent of multidrug resistant (MDR) TB (Aubry et al., 2004). Quinolone drugs have been used primarily for the treatment of patients with multidrug-resistant tuberculosis and are known to target two essential bacterial enzymes, DNA gyrase and topoisomerase IV (Shen et al., 1989). Both enzymes are members of type II topoisomerase wherein DNA gyrase is unique in catalyzing the negative supercoiling of DNA and is essential for efficient DNA replication, transcription and recombination, while topoisomerase IV is a decatenating enzyme that resolves interlinked daughter chromosomes following DNA replication (Hooper, 2001; Hoshino et al., 1994; Kampranis and Maxwell, 1998). However, it appears that mycobacterial genomes lack topoisomerase IV, thus leaving DNA gyrase as the sole type of topoisomerase II in the cell. All mycobacterial species contain complex lipids that have subunits of mycolic acid, which controls the permeability of the antimycobacterial agents (Brennan and Nikaido, 1995). In the face of emerging antibacterial resistance mechanisms, we made an attempt to enhance the efficacy of existing fluoroquinolones, by synthesizing substituted analogues at the 7th position of the quinolone moiety with

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a rationale of increasing their penetration to the lipophilic cell wall of *M. tuberculosis*.

Materials and Methods

Chemicals. Various 7-substituted fluoroquinolone derivatives were prepared as reported in our earlier studies (Sriram et al., 2004). The structures of the synthesized compounds were elucidated using various spectral (IR, ^{1}H-NMR and Mass) and elemental analyses.

Antimycobacterial activity. Alamar blue susceptibility test (MABA): Antimicrobial susceptibility testing was performed in black, clear-bottomed, 96-well microplates (black view plates; Packard Instrument Company, Meriden, Conn.) in order to minimize background fluorescence (Collins and Franzblau, 1997). Outer perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in either dimethyl sulfoxide or distilled deionized water, and subsequent twofold dilutions were performed in 0.1 ml of 7H9GC (no Tween 80) in the microplates. BACTEC 12B-passaged inocula were initially diluted 1 : 2 in 7H9GC, and 0.1 ml was added to wells. Subsequent determination of bacterial titers yielded 1×10^6 CFU/ml in plate wells for *M. tuberculosis* H_{37}Rv. Frozen inocula were initially diluted 1 : 20 in BACTEC 12B medium followed by a 1 : 50 dilution in 7H9GC. Addition of 1/10 ml to wells resulted in final bacterial titers of 2.0×10^5 CFU/ml for *M. tuberculosis* H_{37}Rv. Wells containing drugs only were used to detect autofluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37°C. Starting at day 4 of incubation, 20 μl of 10× alamar Blue solution (Alamar Biosciences/Accumed, Westlake, Ohio) and 12.5 ml of 20% Tween 80 were added to one B well and one M well, and plates were reincubated at 37°C. Wells were observed at 12 and 24 h for a color change from blue to pink and for a reading of 50,000 fluorescence units (FU). Fluorescence was measured in a Cytofluor II microplate fluorometer (PerSeptive Biosystems, Framingham, Mass.) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. If the B wells became pink by 24 h, reagent was added to the entire plate. If the well remained blue or 50,000 FU was measured, additional M and B wells were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37°C, and results were recorded at 24 h post-reagent addition. Visual minimum inhibitory concentration (MIC) was defined as the lowest concentration of drug that prevented a color change. For fluorometric MICs, a background subtraction was performed on all wells with a mean of triplicate M wells. Percent inhibition was defined as 1− (test well FU/mean FU of triplicate B wells)×100. The lowest drug concentration effecting an inhibition of >90% was considered the MIC.

Cytotoxicity assay. Cytotoxicity was assessed against Vero cells (CCL-81, American Type Culture Collection) by exposing monolayers in 96-well plates to 3-fold dilutions of test compounds for 72 h. Cell viability was measured using the CellTiter96 aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, Wis.), which determines the extent of reduction of a tetrazolium dye by measuring the absorbance of the product at 490 nm. Untreated cells and cells lysed with sodium dodecyl sulfate were used to determine 0% and 100% inhibition, respectively.

In-vivo antimycobacterial activity. Mice were infected via low-dose aerosol to reproducibly deliver *M. tuberculosis* in the alveolar regions of the lungs in low numbers to mimic the realistic disease in humans. Treatment was initiated 18 days post infection for 8 daily treatments of one single dose (at 25 mg/kg) (Lenaerts et al., 2003). Bacterial load was determined 28 days post infection in lungs and spleens of the mice by serial dilution of the tissue homogenates on nutrient Middlebrook 7H11 agar plates (GIBCO BRL, Gaithersburg, Md.). The plates were incubated at 37°C in ambient air for 4 weeks prior to the counting of viable *M. tuberculosis* colonies (colony forming unit). The viable counts were converted to logarithms, which were then evaluated by multiple comparison analyses of variance by a one-way Dunnett test using the Sigma Stat program.

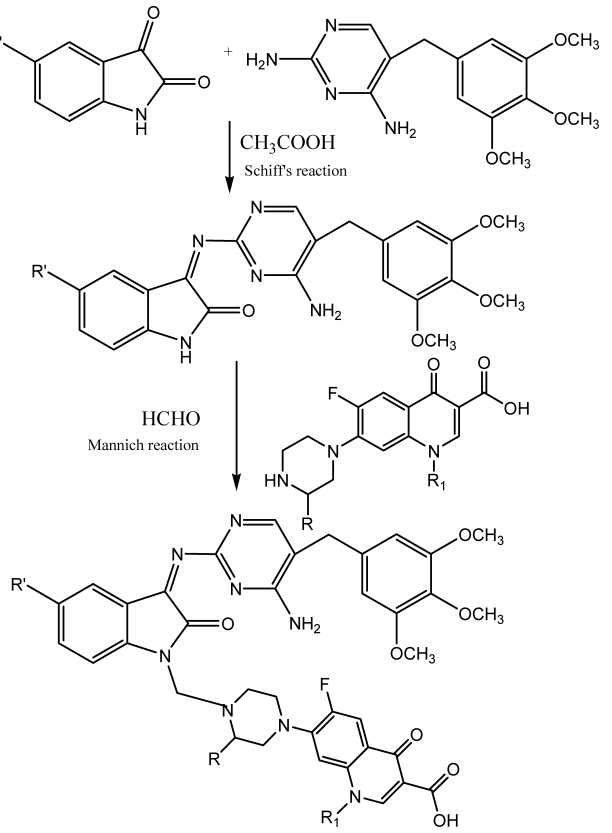
DNA gyrase supercoiling assays. DNA gyrase was purified from *M. smegmatis* cells as described previously (Chatterji et al., 2001). M. Smegmatis SN2 cells were grown in modified Youman and Karlson's medium (Nagaraja and Gopinathan, 1980) to mid-log phase (12–14 h of growth) and harvested. DNA supercoiling assays were carried out by incubating 500 ng of pUC (relaxed DNA) at 37°C in a buffer [35 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 25 mM potassium glutamate, 2 mM spermidine, 2 mM ATP, 50 mg/L bovine serum albumin (BSA), 90 mg/L yeast RNA in 5% (v/v) glycerol]. The

relaxed DNA substrate was prepared by incubating supercoiled pUC 19 DNA with *Escherichia coli* topoisomerase 1 in 40 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$ and 40 mM NaCl for 1 h at 37°C. For assessing the inhibition, the compounds 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 onto 0.8% agarose gels and electrophoresed in TBE buffer for 12 h at room temperature. The gel was then stained with ethidium bromide and DNA was visualized using the BIORAD gel documentation system. All experiments were performed in triplicate.

Results

Synthesis

The synthesis of various 7-substituted fluoroquinolone derivatives was carried out as elucidated in Scheme 1. Isatin and substituted derivatives underwent Schiff's reaction when reacted with 5-(3,4,5-trimethoxybenzyl)-2,4-diamino pyrimidine in the presence of glacial acetic acid (Sriram et al., 2004). The Schiff bases were then treated with various fluoroquinolone derivatives in the presence of formaldehyde via Mannich reaction to yield the desired 7-substituted fluoroquinolones derivatives as illustrated in Table 1. The elemental analysis of the compounds was found to be within ±0.04% of the theoretical values. IR, ^{1}H-NMR spectra and Mass spectra were also consistent with the assigned structures.



Scheme 1. Synthetic protocol of the compounds.

Antimycobacterial activity

All the compounds (F1–F12) exhibited greater than 90% inhibition against *M. tuberculosis* H_{37}Rv strain at 6.25 μg/ml in the primary level screening (Table 2). These selected compounds were further screened at the secondary level at and below 6.25 μg/ml for the determination of MIC value, where the compounds exhibited MIC values ranging from 0.78 μg/ml to 6.25 μg/ml and a selectivity index value between >1.6 to >80.13. Two compounds, F2 and F11, showed promising activity at the secondary level screening with a MIC value of 1.56 and 0.78 μg/ml respectively, and a maximum selectivity index greater than 80.31.

All the compounds were further examined for toxicity (IC_{50}) in a mammalian cell line (Vero cells). Compound

Table 1. Structure and *c* Log *p* of the synthesized fluoroquinolone derivatives (F1–F12).

Comp. No.	R′	R	R_1	R_2	Log *p*
F1	–Cl	–H	–H	△	5.06
F2	–Cl	$–CH_3$	–F	$–C_2H_5$	5.66
F3	–Cl	$–CH_3$	$–OCH_3$	△	5.72
F4	–Br	–H	–H	△	5.30
F5	–Br	$–CH_3$	–F	$–C_2H_5$	5.88
F6	–Br	$–CH_3$	$–OCH_3$	△	5.77
F7	$–CH_3$	–H	–H	△	5.69
F8	$–CH_3$	$–CH_3$	–F	$–C_2H_5$	6.29
F9	$–CH_3$	$–CH_3$	$–OCH_3$	△	6.20
F10	–F	–H	–H	$–C_2H_5$	5.33
F11	–F	–H	–H	△	5.29
F12	–F	$–CH_3$	–F	△	5.86

F11 with a MIC value of 0.78 μg/ml, and selectivity index of more than 80 was not cytotoxic to the Vero cell line up to 62.5 μg/ml.

When evaluated for in vivo antimycobacterial activity, compound F11 was found to decrease the bacterial load in lung tissue (mean colony forming unit of 5.26) and in spleen tissue (mean colony forming unit of 4.08), significantly compared with the counts from controls (untreated) with mean colony forming units of 8.78 and 6.84 in the lungs and in spleen respectively.

DNA gyrase inhibition

Table 3 illustrates the summary of DNA gyrase inhibition at various tested concentrations (10, 20, 40 and 50 μg/ml). Figure 1a represents the supercoiling assays using DNA gyrase obtained from *Mycobacterium smegmatis*. Moxifloxacin was used as the positive control in these assays as it has been shown to be a potent inhibitor of DNA supercoiling by mycobacterial DNA gyrase. The representative data of drugs showing inhibition at lower concentration is also demonstrated in Fig. 1b.

Compound F1, F3 and F6 showed inhibition of the supercoiling reaction catalyzed by the DNA gyrase enzyme at a 50 μg/ml concentration. Compound F7, F9, F10 and F11 did not interfere with the supercoiling reaction at the same concentration, but showed some degree of resistance to this process when compared to the control, i.e. supercoiling in the absence of drugs.

Table 2. Antimycobacterial activity of compounds (F1–F12) by Microplate Alamar Blue Assay test (MABA).

Compound No.	% Inhibition at 6.25 μg/ml	MIC (μg/ml)	IC_{50}(μg/ml) against Vero cells	Selectivity index
F1	100	3.13	>10	>3.19
F2	100	1.56	>10	>6.41
F3	100	3.13	>10	>3.19
F4	100	3.13	>10	>3.19
F5	100	3.13	>10	>3.19
F6	100	3.13	>10	>3.19
F7	100	3.13	>10	>3.19
F8	100	3.13	>10	>3.19
F9	100	3.13	>10	>3.19
F10	100	3.13	>10	>3.19
F11	100	0.78	>62.5	>80.13
F12	100	3.13	>10	>3.19
Isoniazid	100	0.05	>62.5	>1,250
Ethionamide	90	2.5	>62.5	>25
PAS	92	8	>62.5	>7.8
Ethambutol	98	1.88	>62.5	>33.24
Ciprofloxacin	95	2	>62.5	>31.25
Kanamycin	90	5	>62.5	>12.5
Rifampicin	95	0.125	>62.5	>500

Discussion

All the synthesized compounds were initially screened for their antimycobacterial activity at 6.25 μg/ml against the *Mycobacterium tuberculosis* $H_{37}Rv$ strain in BACTEC 12B medium using the mi-

Table 3. Summary of DNA gyrase assay with tested compounds.

Compound No.	Solubility	Inhibition (μg/ml)			
		10	20	40	50
F1	DMSO	No	No	No	Yes
F3	DMSO	No	No	Yes	Yes
F4	DMSO	No	No	No	NC
F6	DMSO	No	No	Yes	Yes
F7	DMSO	—	—	—	No
F9	DMSO	—	—	—	No
F10	DMSO	—	—	—	No
F11	DMSO	—	—	—	No
Moxifloxacin	DMSO	Yes	Yes	Yes	Yes

NC: not clear.

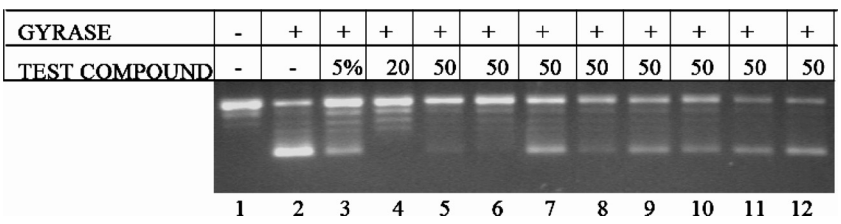


Fig. 1a. Representative data of supercoiling assays.

Lane 1, relaxed substrate; lane 2, supercoiling reaction in absence of drug; lane 3, DMSO control; lane 4, drug control (MFX); lanes 5–12, compounds F1, F3-4, F6-7, F9-11 at 50 μg/ml concentration.

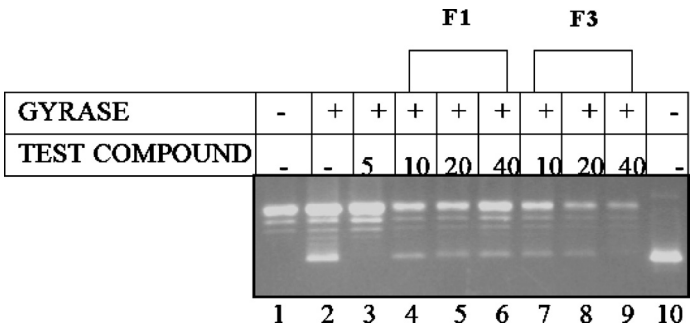


Fig. 1b. Assay with lower concentration of compounds F1 and F3.

croplate alamar blue assay. Compounds exhibiting an inhibition of ≥90% were considered active in these primary assay data, and were retested by serial dilutions (beginning at 6.25 μg/ml) to determine the MIC at the secondary level assay. The compounds were also screened by serial dilution to assess toxicity (IC_{50}) to the Vero cell line. When compared to the standard drugs, compound F11 was found to be more potent (MIC: 0.78 μg/ml) than ethionamide (MIC: 2.5 μg/ml), PAS (MIC: 8 μg/ml), ethambutol (MIC: 1.88 μg/ml), ciprofloxacin (MIC: 2 μg/ml) and kanamycin (MIC: 5 μg/ml), and demonstrated an IC_{50}value of >62.5 μg/ml and selectivity index of >80.31.

Compound F11 was also tested for efficacy against *Mycobacterium tuberculosis*-induced infection of mice at a dose of 25 mg/kg (Table 4) in infected C57BL/6 interferon-γ gene-depleted mice (Lenaerts et al., 2003). In this model, drug treatment began 20 days after inoculation of the animal with MTB. Bacterial counts were measured on day 28 in two tissues, viz. lung and spleen tissues, and compared with the counts from negative (untreated) controls (mean culture forming units (CFU) in lung: 8.78 and in spleen: 6.84). Compound F11 was found to be potent, compared to ciprofloxacin, in reducing bacterial count in the lungs as well as in the spleen.

It is well established that compounds belonging to the quinolone family target the DNA gyrase enzyme to exhibit antibacterial activity. Hence the synthesized fluoroquinolone derivatives were also evaluated in a DNA gyrase supercoiling inhibition assay to verify molecular basis of their antibacterial action. A few of the selected compounds which exhibited significant activity in the secondary level antimycobacterial screening against *Mycobacterium tuberculosis* H_{37}Rv were chosen to test the inhibition of DNA gyrase obtained from *M. smegmatis.* The gyrase subunits from *M. smegmatis* are >90%, similar (Gyr A, 93.7%; Gyr B, 92%) to those present in *M. tuberculosis* at the amino acid level (Chatterji et al., 2001). From the data it is clear that none of the new compounds are as effective as moxifloxacin in inhibiting the supercoiling reaction catalyzed by DNA gyrase but they have bestowed some degree of inhibition. Moreover, F11, which showed more potent activity in in-vitro and in-vivo assay, seems to be a relatively less efficient inhibitor of the enzyme assay conditions.

Table 4. In vivo activity data of F11 and ciprofloxacin against *M. tuberculosis* in mice.

Compound	Lungs (log CFU±SEM)[a]	Spleen (log CFU±SEM)[a]
Control	8.78±0.12	6.84±0.21
F11 (25 mg/kg)	5.26±0.19	4.08±0.11
Ciprofloxacin (25 mg/kg)	6.82±0.18	4.64±0.12

[a] Each value represents mean log CFU±SEM significantly different from the control at $p<0.0001$.

Mycobacteria are gram-positive bacteria that display marked intrinsic resistance to a variety of molecules due to their unique cell wall structure rich in long chain fatty acids composed of mycolic acid. The present study emphasizes the importance of increasing lipophilicity of the compounds to overcome transport barrier into the cells as shown by the increased log *p* values of the synthesized compounds (5.06–6.29), compared to a standard drug like ciprofloxacin (0.01). However, FQ resistance is multifactorial with efflux transporters, cell wall barrier and target site mutations all making important contributions in the development of MDR-TB (Li et al., 2004; Liu et al., 1996; Poole, 2000). Hence, the mechanisms underlying the various other reasons for resistance are to be explored for development of better antitubercular drugs.

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