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Synthesis and antituberculosis activity of novel mefloquine-isoxazole carboxylic esters as prodrugs

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ABSTRACT

5-(2,8-Bis(trifluoromethyl)quinolin-4-yloxymethyl)isoxazole-3-carboxylic acid ethyl ester (compound **3**) was reported to have excellent antituberculosis activity against both replicating and non-replicating *Mycobacterium tuberculosis*, with a minimum inhibitory concentration (MIC) of 0.9 μM and 12.2 μM, respectively. In this study, the antituberculosis activity of compound **3** was further investigated. Its activity appeared to be very specific for organisms of the *M. tuberculosis* complex and it effected significant reductions of bacterial numbers in infected macrophages with an EC_{90} of 4.1 μM. More importantly, the increased in vitro antituberculosis activity of the corresponding acid (compound **4**) at pH 6.0 suggested that it may be active in vivo in an acidic environment produced as a consequence of inflammation in the lungs of TB patients. The fact that various ester bioisosteres of compound **3** lost anti-TB activity further suggested that the ester compound **3** may function as a prodrug. The detailed structure–activity relationships (SARs) from this study should facilitate our ultimate goal of improving the anti-TB potency of this isoxazole ester series.

Tuberculosis (TB) is a major cause of mortality and one of the most frequent AIDS-associated diseases worldwide.[1] The current standard treatment for TB requires a combination regimen of isoniazid (INH), pyrazinamide (PZA), rifampin (RMP), and ethambutol (EMB) for at least six months, which often leads to poor compliance.[2] The difficulty in treating drug-resistant TB, such as multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) also contributes to the increased morbidity and mortality.[3] This demonstrates the urgency of the need for new anti-TB agents.

In our previous work,[4,5] we have explored the SARs of mefloquine-based analogs as anti-TB agents. The modifications included introduction of the hydrazone linker into mefloquine at 4-position, replacement of the piperdine with a piperazine, and extension of the basic terminus of the piperazine ring at the 4-position. Compound **7f**, (Fig. 1), was found to have improved anti-TB activity and selectivity (SI: selectivity index). Moreover, the isoxazole scaffold (Fig. 1) emerged as one of the most potent hits in a high throughput screen of the ChemBridge NovaCore library against *Mycobacterium tuberculosis* in our laboratory. Both compound **7f** and the isoxazole hits consisted of an aromatic ring, and a two-atom linker with either a five or six membered ring. Based on these similarities, compound **3** was designed using a chemical hybridization strategy.[6] Initially, we expected to develop potent anti-TB mefloquine-based ligands with an isoxazolecarboxamide moiety. Instead, 5-(2,8-bis(trifluoromethyl)quinolin-4-yloxymethyl)isoxazole-3-carboxylic acid ethyl ester (compound **3**), was identified, with excellent antituberculosis activity and devoid of detectable cytotoxicity. The minimum inhibitory concentration (MIC) in the microplate Alamar Blue assay (MABA)[7] and the low oxygen recovery assay (LORA)[8] was 0.9 μM (0.4 μg/mL) and 12.2 μM (5.3 μg/mL), respectively, suggesting that compound **3** was potent against both replicating and non-replicating *M. tuberculosis*. The SI of compound **3** (>142.2) was 158-fold higher than that of mefloquine, and it was tolerated at a dosage of ⩾400 mg/kg po in a vehicle of carboxymethylcellulose.

In this study, the antituberculosis activity of compound **3** was further investigated, and various modifications were performed on the ester and the quinoline ring.

It is important to note that *M. tuberculosis* could reside in the lung both intracellularly and extracellularly. While compound **3** was active against *M. tuberculosis* $H_{37}Rv$ and Erdman in axenic medium (Table 1), it also effected a significant reduction in colony forming units (CFU) in *M. tuberculosis*-infected macrophages with an EC_{90} 4.1 μM (1.7 μg/mL) and an EC_{99} 18.3 μM (7.9 μg/mL) (Fig. 2).

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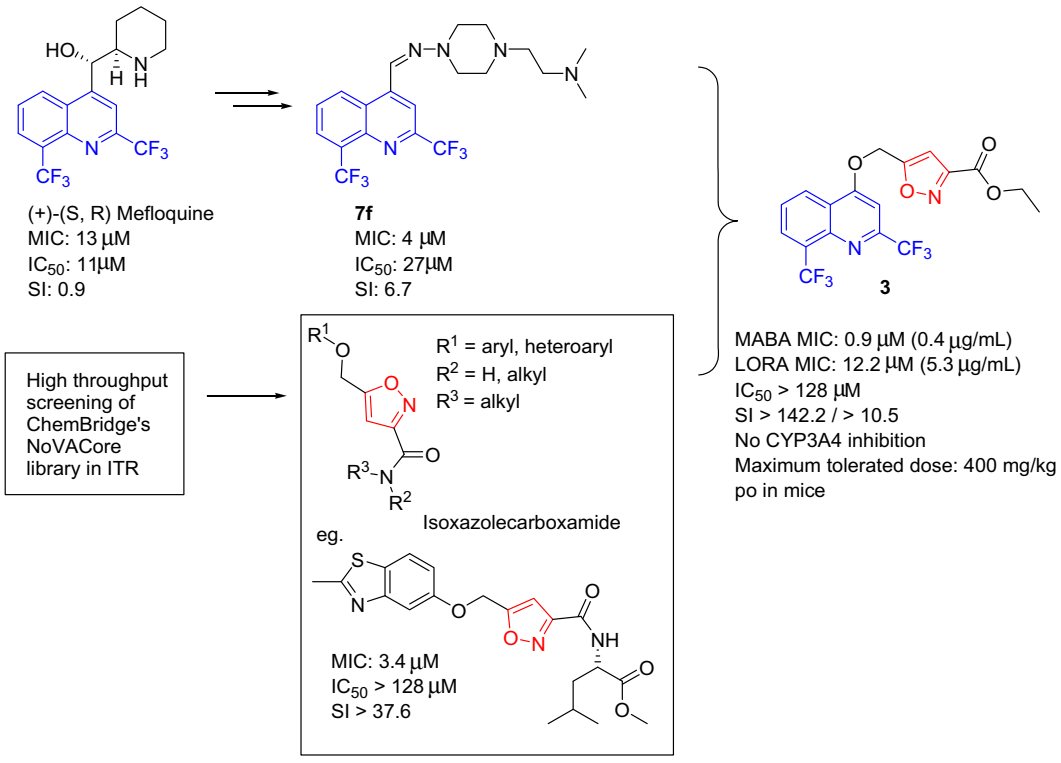
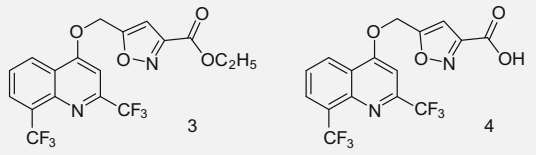


Figure 1. Summary of hybridization design of compound **3**.

Table 1
Antimicrobial spectrum of compounds **3** and **4**



Organism	MIC (μM)	
	3	**4**
Mycobacterium tuberculosis Erdman	1.74	>128
Mycobacterium bovis BCG	<0.5	>128
Mycobacterium smegmatis	>128	>128
Bacillus anthracis	>128	32
Staphylococcus aureus	>128	22
Escherichia coli	>128	>128
Candida albicans	>128	>128

Because of the length of TB treatment, it is preferable that anti-TB agents possess a narrow antimicrobial spectrum. Although compound **4** showed no activity against *M. tuberculosis* $H_{37}Rv$ in our previously study, it may be caused by the poor penetration of acid **4** through the wax-like cell wall of the TB bacterium.[6] For these reasons, compound **3** and its acid **4** were tested against several *mycobacteria* (including *M. tuberculosis* Erdman, *Mycobacterium bovis* BCG and *Mycobacterium smegmatis*), Gram-positive bacteria (*Bacillus anthracis* and *Staphylococcus aureus*), a Gram-negative bacterium (*Escherichia coli*) and a fungus (*Candida albicans*) (Table 1). The activity of compound **3** was very specific for organisms of the *M. tuberculosis* complex, and it showed no activity against the fast grower *M. smegmatis*, which is unusual for an anti-TB agent. Interestingly, although the acid **4** was neither active against *M. tuberculosis* nor the Gram-negative bacteria in vitro, it did demonstrate modest activity against the Gram-positive bacteria. These findings bring to light possible penetration issues of the acid. The acid may be able to penetrate the less hydrophobic cell wall of Gram-positive bacteria but not Gram-negative bacteria nor *mycobacteria*.

It has been reported that weak acids have antituberculosis activity only at acid pH.[9,10] As compound **4** is a weak acid with a calculated pK_a of 3.75, the MICs of compounds **3** and **4** were determined at pH 6.0. As anticipated, the MIC of compound **4** (Table 2) decreased from >128 μM in the normal pH 6.8 culture medium to 7.7 μM in PZA medium (pH 6.0). At the latter pH, the positive control PZA had a reasonable MIC (829 μM, 102.1 μg/mL) comparable to the values reported in the literature.[11] No changes in the MICs of isoniazid (INH) and rifampin (RMP) were observed in response to the acid pH environment. The increased in vitro antituberculosis activity of compound **4** at pH 6.0 suggested that it may be active in vivo as a localized acidic pH environment is likely produced in the lungs of TB patients as a consequence of inflammation.[10] Compound **3** also appeared ninefold more potent in the acidic medium than the neutral pH condition. The acid would be expected to be pumped out by an efflux mechanism, as is the case for pyrazinoic acid (POA, the active form of PZA).[12,13] The charged form of the acid **4** may not be able to penetrate the cell wall at neutral pH. However, at acid pH, some of the neutral form of compound **4** will be present, and thus it should be able to permeate the cell. The higher accumulation of acid **4** may contribute to this ninefold decrease in the MIC of compound **3** at acid pH. This well-correlated decrease in MICs of both compound **3** and compound **4** suggests that compound **3** may function as a prodrug, with the acid as the active form.

In order to further investigate whether compound **3** serves as a prodrug, the ethyl ester was replaced by longer alkyl or aromatic esters. Various esters were prepared from 5-(2,8-bis(trifluoromethyl)quinolin-4-yloxymethyl)isoxazole-3-carboxylic chloride (**6**) with different alcohols at the presence of triethylamine (Scheme 1). All the esters were active against *M. tuberculosis* in

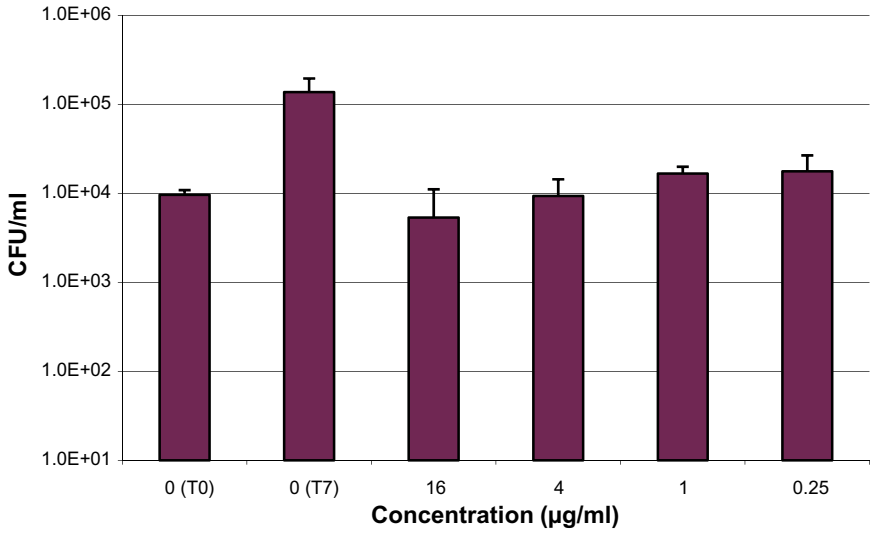


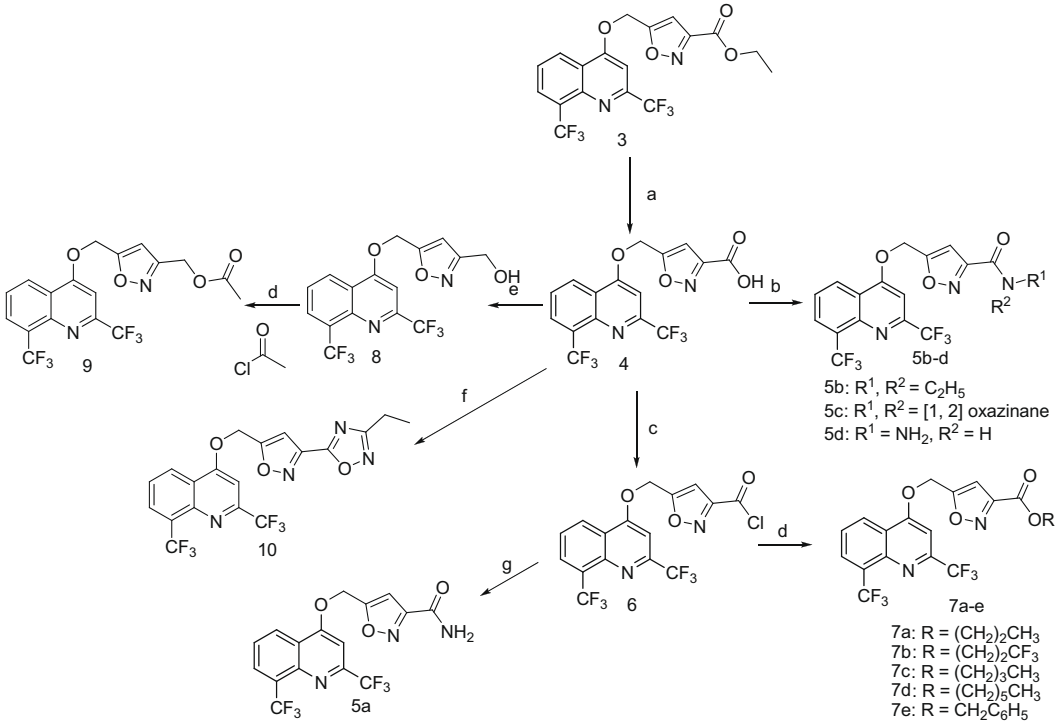
Figure 2. Compound **3** versus *M. tuberculosis* Erdman in J774A.1 macrophages.

Table 2
In vitro antituberculosis activity of compounds **3** and **4** at pH6.8 and pH6.0

Compd	pH 6.8 MIC (μM)	pH 6.0 MIC (μM)
3	0.9	0.1
4	>128 (89%)	7.7
PZA	>4000	829
RMP	0.1	0.2
INH	0.1	0.1

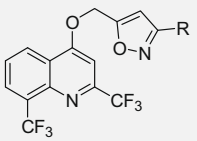
both MABA and LORA. The activities of the propyl ester **7a** (MIC = 1.8/26.1 μM) and the butyl ester **7c** (MIC = 0.9/9.9 μM) were as potent as compound **3** against both replicating and non-replicating *M. tuberculosis* (Table 3). When the methyl group in compound **7a** was replaced by a trifluoromethyl group, compound **7b** with a MIC of 25 μM, was 10-fold less active than that of **7a**. This suggested that electronic effect may play a role in the antituberculosis activity in this series. The putative binding site may have a very limited space as it was observed that esters with a longer alkyl chain (compound **7d**) and an aromatic ring (compound **7e**) had higher MICs (15.3 μM and 26.5 μM, respectively) compared to that of compound **3** in the MABA.

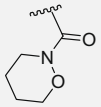
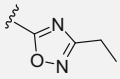
Next, the ester group was replaced by various bioisosteres, such as amides and the oxadiazole. While all the esters demonstrated



Scheme 1. Synthesis of various esters and ester bioisosteres. Reagent and conditions: (a) LiOH, THF/H_2O/MeOH = 3:1:1, rt, 4 h, 90%; (b) RNH_2, EDC, HOBt, iPr_2NH, CH_2Cl_2, rt, overnight; (c) oxalyl chloride, DMF, 15 min, rt; (d) ROH/acetyl chloride, Et_3N, CH_2Cl_2, reflux, overnight; (e) $NaBH_4$, N_2, anhydrous EtOH, overnight; (f) *N*-hydroxypropionamidine, EDC, CH_2Cl_2, reflux, 3 d; (g) NH_4OH, EtOAc, rt, overnight.

Table 3
In vitro activity of esters and bioisosteres against *M. tuberculosis* $H_{37}Rv$



Compd	Structure R=	MIC (μM)		IC_{50} (μM)	SI		Clog P
		MABA	LORA	Vero	MABA	LORA	
1	Mefloquine	13	7	11	0.9	1.6	3.6
RMP	Rifampin	0.1	2	122	1220	61	—
3	$COOCH_2CH_3$	0.9	12.1	>128	>142.2	>10.5	4.4
7a	$COO(CH_2)_2CH_3$	1.8	26.1	>128	>71.2	>4.9	4.9
7b	$COO(CH_2)_2CF_3$	25.0	29.9	>128	>5.1	>4.3	5.3
7c	$COO(CH_2)_3CH_3$	0.9	9.9	105.9	>109.1	>10.6	5.4
7d	$COO(CH_2)_5CH_3$	15.3	26.7	>128	>8.4	>4.8	6.4
7e	$COOCH_2C_6H_5$	26.5	50.7	>128	>4.8	>2.5	5.6
5a	$CONH_2$	>128	>128	>128	—	—	3.5
5b	$CON(C_2H_5)_2$	>128	>128	46.5	—	—	4.4
5c		>128	>128	>128	—	—	4.6
5d	$CONHNH_2$	>128	>128	>128	—	—	2.5
8	CH_2OH	>128	>128	>128	—	—	3.2
9	CH_2OCOCH_3	>128	>128	>128	—	—	4.2
10		>128	>128	>128	—	—	4.6

some anti-TB activity, none of the ester bioisosteres (compounds **5a**–**d** and **9**–**10**) were active.

As INH and PZA are known to serve as prodrugs, the amide and hydrazide moieties were introduced to the isoxazole carboxylic acid. Compound **5a** was prepared from 5-(2,8-bis(trifluoromethyl)quinolin-4-yloxymethyl)isoxazole-3-carboxylic acid chloride (**6**) by reaction with concentrated NH_3 (aq) (Scheme 1). Compound **5d** was prepared from the carboxylic acid **4** by reaction with hydrazine monohydrate. However, neither **5a** nor **5b** was active against *M. tuberculosis* (Table 3).

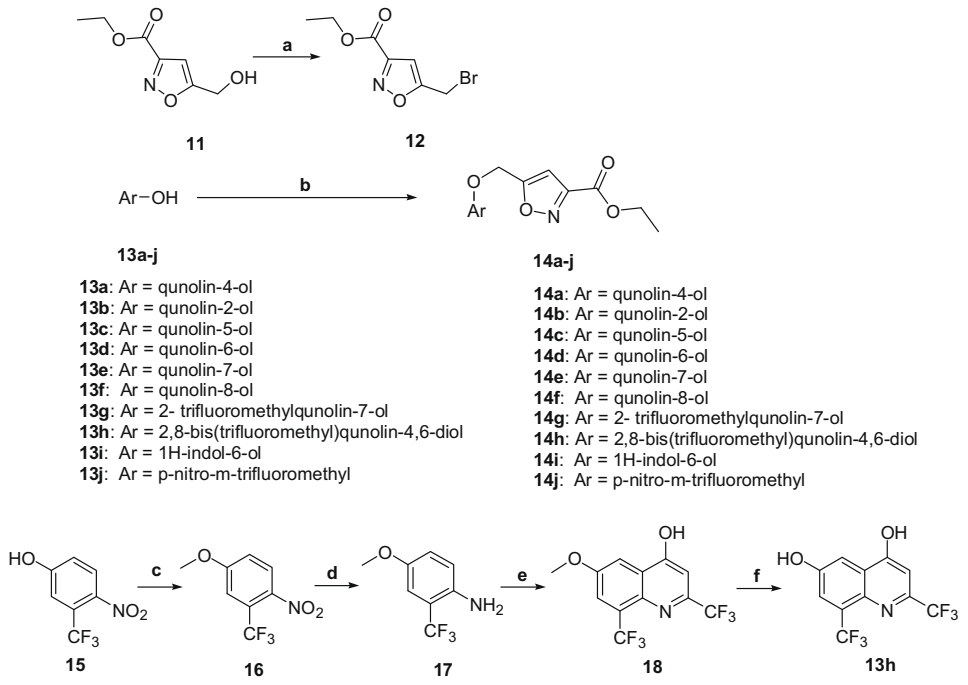
Two tertiary carboxamides were prepared which included the diethylamide **5b** and the [1,2]oxazinane-2-carbonyl **5c** as they might be cleaved more easily. Both tertiary carboxamides were prepared from the acid **4**, which was reacted with the appropriate amines in the presence of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). Unfortunately, compound **5b** and **5c** failed to show any anti-TB activity. In summary, none of amides were active against *M. tuberculosis* including primary (compound **5a**), secondary (compounds in Ref. 6) and tertiary amides (compounds **5b** and **5c**).

The acid **4** was reduced to its alcohol form **8** by sodium borohydride, from which a new ester **9** was generated. The extended ester **9** failed to show any in vitro activity against *M. tuberculosis* as did the intermediate alcohol **8**.

It is known that [1,2,4]oxadiazoles are able to serve as ester bioisosteres, and therefore this heterocycle was introduced into our lead compound by the reaction of the acid **4** and *N*-hydroxypropionamidine in the presence of EDC (Scheme 1). As with the other bioisosteres, compound **10** also failed to show any anti-TB activity.

That none of the ester bioisosteres were active against *M. tuberculosis* in vitro further supported the hypothesis that **3** functions as a prodrug. The inactivity of the alcohol **8** and the isomeric ester **9** ruled out the possibility of the alcohol of compound **3** serving as the active metabolite. Thus, in advancing the SAR work from this point forward, we decided to maintain the ethyl ester moiety and focus our further modifications on the quinoline ring.

Inspired by the SAR from the Novacore library (unpublished data), the position of the oxymethylene linker on the quinoline ring was investigated. Ideally, the SAR could be built around the 2,8-bis(trifluoromethyl)quinoline as the key scaffold. Although the hydroxyquinoline **14h** was prepared by the traditional Combes quinoline synthesis (Scheme 2), the route was somewhat lengthy and the yield of quinoline ring formation was low. Therefore, instead of employing 2,8-bis (trifluoromethyl)quinoline as the start-



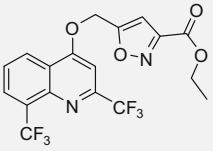
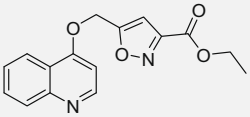
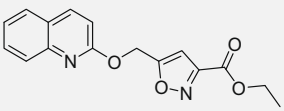
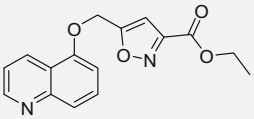
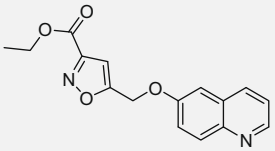
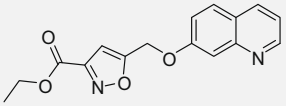
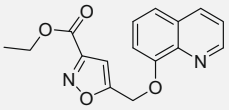
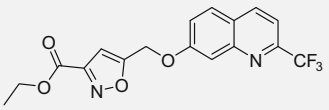
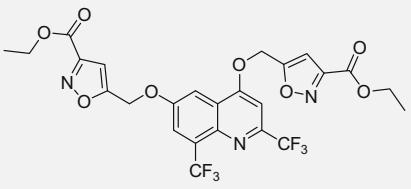
Scheme 2. Synthesis of compounds in Tables 4 and 5. Reagent and conditions: (a) CBr_4/PPh_3, CH_2Cl_2, −15 °C, 2 h; (b) **12**, K_2CO_3, acetone, reflux, overnight; (c) MeI, K_2CO_3, acetone, reflux, overnight, 95%; (d) Zn/HCl, MeOH, 90%; (e) ethyl 4,4,4-trifluoroacetoacetate, PPA, 40%; (f) 48% HBr, reflux, overnight, 95%.

ing point of the SAR, the unsubstituted hydroxyquinoline was used instead, as many of the starting materials are commercially available.

Compounds **14a**–**j** were prepared from various hydroxyquinolines by reaction with ethyl 5-(bromomethyl)-3-isoxazolecarboxylate (**12**), which is the bromination product of 5-hydroxy methylisoxazole-3-carboxylic acid ethyl ester (Scheme 2). Compound **14a** (MABA MIC = 25.1 μM) was more than 26-fold less active than compound **3**, although it had similar MIC in the LORA assay (11.3 vs 12.2 μM, respectively) (Table 4). This suggested that the 2- and 8-trifluoromethyl groups on the quinoline ring were essential for the antituberculosis activity against replicating *M. tuberculosis*. In the MABA, compound **14b** (MIC = 103.3 μM), **14c** (MIC = 35.1 μM), and **14f** (MIC = 39.8 μM) with the oxymethylene linker attached to the 2-, 5-, and 8-positions of the quinoline ring, respectively, were less active than the 4-substituted compound **14a** (MIC = 25.1 μM). However, the MICs of compounds **14d** (7.4 μM) and **14e** (10.8 μM) with oxymethylene linkers attached to the 6- and 7-positions, respectively, decreased 2–3-fold compared with that of compound **14a.** We expected that introduction of a trifluoromethyl group into the quinoline of **14e** to improve its MIC value. Indeed, the 2-trifluoromethyl substituted compound **14g** (MABA MIC = 3.6 μM) with an oxymethylene linker attached to the 7-position was threefold more active than the unsubstituted compound **14e**.

As the 4-, 6-, and 7-positions of quinoline were promising positions for attachment of the oxymethylene linker, the capacity of the binding site was further explored. We assume that for the active compounds **14a**, **14d** and **14e**, the interaction between the isoxazole ester moiety and the active site of the putative target is important for activity. And it is likely that the isoxazole carboxylic ester groups in these compounds bind to the same binding pocket

Table 4
In vitro activity of various quinolines against *M. tuberculosis* $H_{37}Rv$

Compd	Structure	MIC (μM)		IC_{50} (μM) Vero	SI		*Clog P*
		MABA	LORA		MABA	LORA	
1	Mefloquine	13	7	11	0.9	1.6	3.6
RMP	Rifampin	0.1	2	122	1220	61	—
3		0.9	12.2	>128	>144	>10.5	4.4
14a		25.1	11.3	>128	>5.1	>11.3	2.5
14b		103.3	64	>128	>1.2	>2	2.5
14c		35.1	31.1	>128	>3.6	>4.1	2.5
14d		7.4	12.8	>128	>17.3	>10	2.5
14e		10.8	40.3	107.6	>9.9	>2.7	2.5
14f		39.8	125.1	>128	>3.2	> 1.0	2.5
14g		3.6	21.6	>128	>25.6	>5.9	3.8
14h		17.6	15.6	>128	>7.3	>8.2	5.6

while the orientations of the quinoline rings may be different. To confirm this assumption, compound **14h** which contains two such oxymethylene linked isoxazole groups at 4- and 6- positions was designed and synthesized.

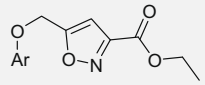
Compound **14h** was prepared in five steps from 4-hydroxy-1-nitro-2-trifluoromethylbenzene (**15**) (Scheme 2). The hydroxyl group was protected by a methyl group, and then compound **16** was reduced using Zn–HCl to afford 4-methoxy-2-trifluoromethyl-phenylamine (**17**). The Combes quinoline synthesis was carried out by reacting compound **17** with ethyl 4,4,4-trifluoroacetoacetate in the presence of polyphosphoric acid (PPA). The product **18** was demethylated to give compound **13h**, which was reacted in turn with ethyl 5-(bromomethyl)-3-isoxazolecarboxylate to afford 2,8-bis(trifluoromethyl)quinolin-4,6-bis(5-methoxyl-isoxazole-3-carboxylic acid ethyl ester) (**14h**). The MABA MIC of compound **14h** was 17.6 μM; 18-fold higher than that of compound **3**. If there were more than one binding pockets for isoxazole carboxylic esters, the 4,6-disubstituted compound **14h** should show comparable activity with that of **3**. The remarkably decreased activity of **14h** is consistent with our assumption that the isoxazole carboxylic acid ester groups of different active compounds bind to the same binding pocket.

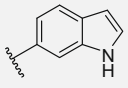
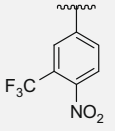
As discussed above, compared to the isoxazole carboxylic ester part, the quinoline ring may not be crucial for activity. To further explore the importance of the quinoline ring, it was replaced by *1H*-indole and phenyl rings, and compounds **14i–j** were prepared by the same synthetic route as compound **14a** (Scheme 2).

Both compounds **14i** (MABA MIC = 8.6 μM) and **14j** (MABA MIC = 3.5 μM) were shown to be more active in vitro than the corresponding quinoline compound **14e** (MABA MIC = 10.8 μM). When **14e** and **14i–j** were compared structurally, it was found that the phenyl part in the quinoline group of **14e** was conserved in **14i–j** while the pyridine part in the quinoline group was downsized (**14i**) or completely removed (**14j**). This suggests that among the two rings in the quinoline moiety, the phenyl ring may play a more important role than the pyridine ring against replicating *M. tuberculosis*.

As a summary, compound **3** was found to be active against *M. tuberculosis* both intracellularly and extracellularly, and its activity was specific for members of the *M. tuberculosis* complex. Various ester analogs were active against *M. tuberculosis* in both MABA and LORA. The compounds with small ester substituents, such as propyl and butyl esters (compound **7a** and **7c**), were as active as the lead compound **3**. However, none of the ester bioisosteres, including the isomeric ester, amides, and the oxadiazole analogs, showed any activity. These observations suggested that the ester

Table 5
In vitro activity of various aryl substituents against *M. tuberculosis* $H_{37}Rv$



Compd	Structure Ar=	MIC (μM)		IC_{50} (μM)	SI		Clog P
		MABA	LORA	Vero	MABA	LORA	
1	Mefloquine	13	7	11	0.9	1.6	3.6
RMP	Rifampin	0.1	2	122	1220	61	—
14i		8.6	42.9	>128	>14.9	>2.9	2.4
14j		3.5	62.2	>128	>36.6	>2.1	3.6

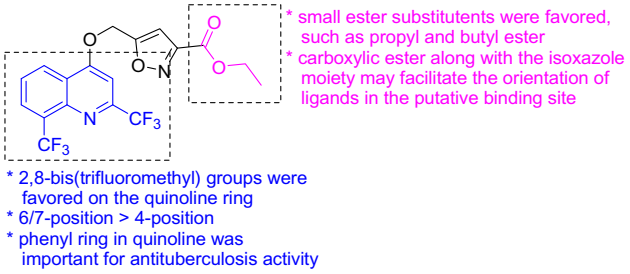


Figure 3. SARs on compound **3**.

may be acting as a prodrug. The well-correlated improved antituberculosis activity of the acid **4** and compound **3** at acid pH further support this novel hypothesis. As the acidic pH environment is possibly produced as a consequence of inflammation in the lungs of TB patients, the acid may be active in vivo. In this context, our modifications were focused on the quinoline ring and positions of the oxymethylene linker substitution. 2,8-Bis(trifluoromethyl) substitutions on the quinoline were found to be favored, and the phenyl ring in the quinoline was important for its antituberculosis activity. The 6- and 7-positions were better than the 4-position for location of the oxymethylene linker. It is also suggested that the isoxazole carboxylic acid ester moiety forms a key interaction with the active site of the putative target, which is crucial for activity, while the quinoline ring may be less important (Fig. 3). As the SAR results in the present study support the idea that compound **3** may function as a prodrug, the metabolism of compound **3** has been studied, and these results then used to design and synthesize a new series of mefloquine-isoxazole carboxylic acid esters with better in vitro and in vivo metabolic stability.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.11.105.

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