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Discovery of Novel Isoxazolines as Anti-tuberculosis Agents

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Abstract

Nitrofuranyl isoxazolines with increased proteolytic stability over nitrofuranyl amides were designed and synthesized leading to discovery of several compounds with potent *in vitro* anti-tuberculosis activity. However, their *in vivo* activity was limited by high protein binding and poor distribution. Consequently, a series of non-nitrofuranyl containing isoxazolines was prepared to determine if the core had residual anti-tuberculosis activity. This led to the discovery of novel isoxazoline **12** as anti-tuberculosis agent with a MIC₉₀ value of 1.56 µg/mL.

Keywords

Isoxazolines; Nitrofurans; Anti-tuberculosis agents

Mycobacterium tuberculosis is a very successful pathogen that infects one third of the world's population.¹ The emergence of multi-drug resistant tuberculosis and extensively drug resistant tuberculosis coupled with an increasing number of tuberculosis patients due to the overlap between tuberculosis and AIDS epidemics has created an urgent need to develop novel therapeutics to treat this deadly disease.¹ In order to develop a better chemotherapeutic regime it is believed that drugs are most needed to treat the latent phase of this disease. Unfortunately, latent bacteria are intrinsically more difficult to treat.² The nitroaromatic class of antibiotics is one of the few classes of antibiotics that have shown activity against latent *M. tuberculosis*, and nitroimidazoles PA-824 and OPC-67683 are in current clinical trials to treat tuberculosis.³ We chose to investigate a related class, the nitrofurans. Previously, we discovered and developed a series of nitrofuranyl amides with excellent *in vitro* activity against *M. tuberculosis* (Figure 1).⁴ However, this series of compounds did not perform well during *in vivo* studies due to a short biological half life and rapid elimination. The amide linkage (shown in green, Figure 1) was thought to be the major reason for the observed metabolic instability. Thus in this current study we evaluated the replacement of the amide linker with an isoxazoline linker (shown in pink, Figure 1). The isoxazoline ring system represents a stable bioisosteric replacement for the amide bond that is found among many biologically active molecules and drugs.⁵

The synthesis of the nitrofuranyl isoxazoline compounds is shown in Scheme 1. First, the olefin **2** was prepared in good yield (88%) by a palladium-catalyzed aromatic amination reaction on

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p-bromo styrene **1** with *N*-Boc-piperazine.⁶ Second, to establish the isoxazoline bridge, the nitrile oxide was generated *in situ* from oxime **4** following Torsell's procedure, which upon treatment with olefin **2**, underwent a [3+2] regioselective cycloaddition⁷ to give isoxazoline **5** in 67% yield.⁸ Boc-deprotection of **5** was achieved by aqueous trifluoroacetic acid treatment to yield the free amine. The free amine was treated with benzyl bromide in the presence of K₂CO₃ to afford **6a** (59%). Compounds **6b** and **6c** were obtained by reacting the free amine with ethyl chloroformate and isopropyl isocyanate (82% and 86%) respectively.⁹ **7** was synthesized in a similar manner to compound **5** starting by reacting **1** and piperidine to form **3** (86% yield) and then reacting **3** with **4** to give isoxazoline **7** (63% yield).

The anti-tuberculosis activity of compounds **5**, **6a–c**, and **7** were tested using microbroth dilution (Table 1).¹⁰ All compounds in this series demonstrated outstanding MIC activity and compounds **6a–c** were advanced for *in vivo* testing in a short term mouse model of tuberculosis infection.¹¹ Unfortunately, only modest reduction in the bacterial load was observed after a 9 day treatment regime (Table 1) ($P < 0.05$). This led us to more closely examine the biopharmaceutic and pharmacokinetic properties of the series. Solubility was determined at two different pH values using a miniaturized shake-flask method.¹² Metabolic stability of the compounds was assessed in pooled rat liver microsomal preparations by monitoring disappearance of the compound. The percentage of intact parent compound was estimated using an LC-MS/MS assay. Plasma protein binding was determined by equilibrium dialysis using RED® devices (Pierce Biotechnology Inc, Rockford, IL). The results of these studies are also included in Table 1. Compound **6a** was selected for further *in vivo* evaluation of pharmacokinetic properties in rats. **6a** was found to have an oral bioavailability of about 35%, an acceptable elimination half-life about 2.6 hours but a relatively small volume of distribution of 2.0 L/Kg.¹³ The *in vivo* efficacy of anti-infective agents is usually dictated by their intrinsic antimicrobial activity and their free, unbound concentration in the target tissue, as only free, non-protein bound drug is pharmacologically active. Thus these results seem to suggest that the limiting factor for these highly protein bound compounds is tissue penetration.

The outstanding anti-tuberculosis potency of **5–7** led us to question if the core isoxazoline scaffold itself had any intrinsic anti-tubercular activity. To test this hypothesis, a subsequent set of isoxazoline compounds was synthesized keeping the main core but altering the nitrofuranyl portion (Scheme 2) using a similar synthetic strategy. First, the *p*-bromostyrene was subject to a palladium catalyzed amination reaction with benzylpiperazine under the similar conditions described earlier to give the olefin intermediate **9** in 79% yield. Then **9** was reacted with different oximes in the presence of NaOCl and catalytic triethylamine to give corresponding 3+2 cycloaddition products **10a–d** in 45–60% yields. The ester analog was created by reacting **9** with the commercially available building block **11** in the presence of base triethylamine to afford isoxazoline ethyl ester derivative **12** in 71% yield.¹⁴

The anti-tuberculosis activity of this series was determined and is shown in Table 2. Compound **12** was most active with a MIC₉₀ of 1.56 µg/mL against *M. tuberculosis*. The remainder of the compounds **10a–d** did not show any appreciable activity. Importantly, **12** represents a novel isoxazoline chemotype for which anti-tuberculosis properties have not been previously noted.

In conclusion, isoxazoline linked nitrofurans were synthesized. These compounds had better anti-tuberculosis activity *in vitro* and had improved serum half lives over corresponding compounds in the previous nitrofuranyl amide series, demonstrating that the strategy of replacing the amide bond with isoxazoline ring was successful.⁴ However the series still possessed limited *in vivo* efficacy. A detailed pharmacokinetic analysis of these agents showed them to be limited by low solubility, high serum protein binding and a low volume of distribution. When these results are combined it strongly suggests that *in vivo* efficacy is limited by poor tissue penetration and low concentrations of free drug at the site of infection. These

factors are now being addressed in the design of the next generation of compounds in this series.

As the nitrofuranyl isoxazole series was so potent *in vitro* we explored if the core isoxazoline had any intrinsic anti-tuberculosis activity. This led to the discovery of a novel isoxazoline compound **12** with MIC₉₀ value of 1.56 µg/mL. This is a new chemotype though less potent than the nitrofurans it does offer some significant potential advantages including increased solubility as the compounds are less crystalline and lower potential side effects as no nitro group is present. Further optimization of this series is ongoing and will be reported subsequently.

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8. Sammelson RE, Miller RB, Kurth MJ. J Org Chem 2000;65:2225. [PubMed: 10774051] General procedure for 3+2 cycloaddition to form isoxazoline linkage: Olefin (1.0 eq), aldoxime (1.0–1.5 eq) and Et₃N (0.2 eq, catalytic) were dissolved in DCM, and the solution was cooled to 0 °C. Bleach containing 5% NaOCl by weight (3–6 eq) was added dropwise to the vigorously stirring solution. The biphasic mixture was allowed to warm to room temperature and stirred for 8 h overnight. An additional volume of water (equal to the volume of bleach) was added and the layers were separated. The aqueous layer was extracted two to three additional times with DCM, and the combined organic layers were dried with sodium sulfate, rota-evaporated and flash column purified to give the product as

enantiomeric mixture. In case of poor solubility of oxime such as nitrofuranyl oxime a 1:1 mixture of DCM and THF was used as reaction solvent. In this case, for workup, after the reaction the reaction mixture was concentrated on rota-evaporator and the crude product obtained was subjected to water work up. Caution: Nifuraxime is light sensitive hence the proper precautions have to be taken during weighing, transferring etc.

9. Representative analytical data of compound **6a**. ¹H NMR (500 MHz, CDCl₃): δ 2.5–2.62 (4H, broad s), 3.1–3.23 (4H, m), 3.32 (1H, dd, J = 9.0, 17.3 Hz), 3.53 (2H, s), 3.66 (1H, dd, J = 11.2, 17.3 Hz), 5.66 (1H, dd, J = 9.0, 11.2 Hz), 6.83 (2H, d, J = 8.7 Hz), 6.95 (1H, d, J = 3.9 Hz), 7.14–7.24 (3H, m), 7.25–7.33 (5H, m); ¹³C-NMR (300 MHz, CDCl₃): ppm 29.14, 40.59, 48.05, 52.27, 62.35, 83.56, 111.82, 112.47, 115.39, 126.59, 127.82, 128.79, 128.97, 146.84, 147.27, 151.08; ESI MS: 455.2 (M + 23). HPLC purity: 100%, *t*_R = 5.23 min.
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13. Pharmacokinetic studies of compound **6a**

Route	<i>t</i> _{1/2} (hr)	AUC _∞ (μg hr/L)	Volume of distribution (L/kg)	Clearance (L/hr/kg)
IV (10mg/kg)	2.6	19091	2.0	0.53
Oral (100 mg/kg)	4.2	65931	9.3	1.58

14. Synthetic procedure for compound **12**. To a stirred mixture of olefin **9** (0.2 g, 0.719 mmol) and Et₃N (0.2 mL, 1.438 mmol) in anhydrous CH₂Cl₂ (10 mL) ethyl chloroximido acetate (0.163 g, 1.079 mmol) was added in portions at 0 °C. The reaction mixture was stirred at RT for 8 h and washed with water (2 × 10 mL), dried (anhyd. Na₂SO₄) and concentrated under reduced pressure. The crude product was purified by flash chromatography to give **12** (0.2 g, 71%) as a yellow solid. ¹H NMR (500 MHz, CDCl₃): δ 1.37 (3H, t, J = 7.0 Hz), 2.6 (4H, t, J = 4.8 Hz), 3.17–3.25 (5H, m), 3.5–3.51 (3H, m), 4.36 (2H, q, J = 7.3 Hz), 5.7 (1H, dd, J = 9.2, 11.4 Hz), 6.89 (2H, d, J = 8.7 Hz), 7.21 (2H, d, J = 8.7 Hz), 7.25–7.29 (2H, m), 7.31–7.37 (3H, m); ¹³C NMR (500 MHz, CDCl₃): δ 14.24, 40.84, 48.73, 52.98, 62.07, 63.06, 85.33, 115.79, 127.22, 127.31, 128.35, 129.23, 129.56, 138.01, 151.28, 151.69, 160.78; MS: 394.4 (M+1); HPLC purity: 100%, *t*_R = 5.13 min.

Figure 1. Discovery of novel isoxazoline compound in the course of developing nitrofuran anti-tuberculosis agents.

Scheme 1. Synthesis of nitrofuranyl derivatives with an isoxazoline linker

^aReagents and conditions: a) *N*-Boc piperazine or piperidine, PdCl₂[P(*o*-tol)₃], NaO^tBu, Toluene, 100 °C, 3 h; b) *N*-chlorosuccinimide, pyridine, dry Et₃N, CHCl₃, 60 °C - rt, 2 h; c) CF₃COOH-H₂O, THF, rt; d) BnBr, K₂CO₃, DMF, rt, 6 h; e) EtOCOC₂H₅, Et₃N, THF, rt, 6 h; f) ⁱPrNCO, Et₃N, THF, rt, 6 h.

Scheme 2. Synthesis of Isoxazoline compounds by altering the nitrofurant motif

^aReagents and conditions: a) $\text{PdCl}_2[\text{P}(o\text{-tol})_3]$, NaO^tBu , Toluene, 100 °C, 3 h; b) Oxime, 5% NaOCl , cat. Et_3N , CH_2Cl_2 , rt, c) Et_3N , CH_2Cl_2 , rt.

Table 1
Anti-tuberculosis activity and in vitro data of nitrofurans compounds with isoxazoline linkage (NT:Not Tested)

Compd.	Structure	<i>M. tb</i> MIC ₉₀ (μg/mL)	Solubility (mg/L) pH 6.0 pH 7.4	MW (g/ mol)	cLogP	% Protein bound	Microsomal stability % remaining@ 90min	Log ₁₀ Reduction in <i>M. tb</i> CFU in Lung vs. untreated controls± SEM
5		0.0001	NT NT	442.5	3.99	NT	NT	NT
6a		0.00005	3.34 0.236	432.5	4.29	99.9	31	0.64 ± 0.21
6b		0.0001	4 3.6	414.4	3.28	99.0	12	0.59 ± 0.22
6c		0.0002	27.7 20	427.5	2.58	97.3	26	0.83 ± 0.20
7		0.00156	0.017 0.014	341.4	4.48	98.8	6	NT
Isoniazid		0.025	NT NT	137.1	-0.67	NT	NT	3.98 ± 0.26

Table 2
Anti-tuberculosis activity of isoxazolines **10a–d** and **12**

Compd.	Structure	MIC ₉₀ (μg/mL)
10a		>200
10b		>200
10c		50
10d		>200
12		1.56