Table 8. Effect of OPC-67683 on CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 Mediated Reactions in Human Liver

CYP	Reaction	OPC-67683 or In hibitor	Percent of Control		
			Concentration (μM)		
			10	30	100
CYP1A1/2	7-Ethoxyresorufin O-deethylation	OPC-67683	98.4	102.5	98.6
		Furafylline			32.3
		7,8-Benzoflavone			3.8
CYP2A6	Coumarin 7-hydroxylation	OPC-67683	103.1	97.8	100.8
		Diethyldithiocarbamate			38.2
CYP2B6	7-Benzyloxyresorufin O-debenzylation	OPC-67683	118.3	112.8	122.3
		Orphenadrine			118.8
CYP2C8/9	Tolubutamide methylhydroxylation	OPC-67683	107.2	107.8	108.5
		Sulfaphenazole			25.5
		Quercetin			30.6
CYP2C19	S-mephenytoin 4′ -hydroxylation	OPC-67683	113.3	106.5	107.6
		Tranylcypromine			16.6
CYP2D6	Bufuralol 1′ -hydroxylation	OPC-67683	99.1	103.3	97.8
		Quinidine			0.0
CYP2E1	Chlorzoxazone 6-hydroxylation	OPC-67683	110.8	112.4	112.5
		Diethyldithiocarbamate			55.0
CYP3A4	Testosterone 6β-hydroxylation	OPC-67683	117.7	117.7	115.6
		Ketoconazole			0.5
CYP3A4	Nifedipine oxidation	OPC-67683	101.3	99.9	100.3
		Ketoconazole			3.4

The substrate concentrations used for each assay were 0.5 μM 7-ethoxyresorufin, 2 μM coumarin, 1.5 μM 7-benzyloxyresorufin, 400 μM tolbutamide, 100 μM S-mephenytoin, 20 μM bufuralol, 100 μM chlorzoxazone, 100 μM testosterone, and 50 μM nifedipine. Enzyme incubations and metabolite analysis were carried out in triplicate. Each value represents the mean.
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bioavailability of the drug itself as well as other CYP-intermediated drugs, including protease inhibitors, which are indispensable in the treatment of HIV/AIDS [37]. It is therefore important that a new TB drug does not induce nor is affected by metabolic enzymes. With this in mind, we studied the interactions between OPC-67683 and metabolic enzymes. Our results showed that OPC-67683 was hardly metabolized when exposed to human and animal liver microsomes and did not have inductive, stimulatory, or inhibitory effects on CYP enzyme activities at concentrations up to 100 μM, indicating that OPC-67683, at the expected therapeutic concentrations, would not be expected to cause clinically significant interactions with other CYP-metabolized drugs, such as rifamycin derivatives. These results, together with data supporting non-compromised anti-TB activity in immunodeficient animals, suggest that OPC-67683 could be useful in treating TB patients who are also co-infected with HIV/AIDS.

We conclude that OPC-67683 possesses qualities that could help address the unmet needs in TB chemotherapy, i.e., the need for shortened treatment duration, effectiveness against MDR-TB, ability to be used safely in HIV/AIDS patients, and the treatment of LTBI. An early Phase II clinical study to confirm the efficacy in patients is now ongoing.

Furthermore, the Global Alliance for TB Drug Development is aiming to establish an entirely new regimen containing the best combination of new drugs [38]. Development and integration of these drugs into the regimen individually would normally be done in series, taking at least six years for each drug. We therefore attach importance to including an evaluation of the effects of OPC-67683 in combination with not only conventional drugs but also new drugs as early as possible in order to contribute data necessary for establishing the best regimen needed to address the unmet needs in TB treatment.

Supporting Information

Table S1. Viable Count in Lung of Each Group of OPC-67683, RFP, INH, EB, SM, PZA, and PA-824 after 4 wk of Treatments on the Experimental Chronic TB Model in Mice

Found at doi:10.1371/journal.pmed.0030466.st001 (43 KB DOC).

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Author contributions. All listed authors actively participated in the studies related to OPC-67683 described in this manuscript. M. Matsumoto established a strategy for screening for all synthesized compounds, and was instrumental in selecting and evaluating OPC-67683 through conducting susceptibility tests, establishing the inhibitory activity of OPC-67683 on mycolic acid biosynthesis, and carrying out all in vivo studies involving OPC-67683 in collaboration with H. Hashizume, T. Tomishige, and M. Kawasaki. H. Hashizume was responsible for conducting the bacteria reverse mutation testing and the absorption study in mice. T. Tomishige looked after determining the intracellular activity of OPC-67683 and confirming the potency in the immunosuppressive animal model. M. Kawasaki conducted the studies related to the mechanism of action, susceptibility testing, experimental isolation of resistant strains, confirmation of a mutation in the Rv3547 gene in OPC-67683-resistant strains, and identification of metabolites. H. Tsubouchi and M. Komatsu coordinated the overall activities involved in synthesizing the many

novel derivatives for selecting potent antituberculosis agents, and, together with H. Sasaki, synthesized and supplied the derivatives used for in vitro and in vivo evaluations. They also established the facile and practical synthesis method for the intermediates to synthesize many target compounds and supplied derivatives for the screening toxicity test in animals in large scale. H. Sasaki assumed a main role in synthesising various compounds, including OPC-67683. Y. Shimokawa was in charge of the drug interaction studies.

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Editors' Summary

Background. One-third of the world's population is infected with *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis (TB). Most infected people are healthy—the bacteria can remain latent for years, hidden within cells in the body. However, every year 8 million people develop active TB, a chronic disease that usually affects the lungs, and 2 million people die. For most of the second half of the 20th century, TB was in decline because of the powerful antibiotics that were developed from the 1940s onwards. The standard treatment for TB—four antibiotics that have to be taken several times a week for at least six months to flush out any latent *M. tuberculosis* bacteria—was introduced in the late 1970s and saved many lives. Recently, however, efforts to eradicate TB have been set back by the HIV/AIDS epidemic—people with damaged immune systems are very susceptible to TB—and the emergence of multi-drug resistant (MDR) bacteria.

Why Was This Study Done? The treatment for TB is long and unpleasant, and patients who develop MDR-TB have to be treated with second-line drugs that are less effective, more expensive, and more toxic. In addition, for people infected with both HIV and TB, some antiretroviral and anti-TB drugs cannot be used at the same time. Many drugs are either activated or removed by enzymes in the liver, so combinations of these two classes of drugs sometimes alter liver function in a way that causes clinical problems. There is, therefore, an urgent need for new, effective anti-TB drugs that attack *M. tuberculosis* in a different way than do existing drugs. Such drugs should ideally be active against MDR *M. tuberculosis*, work quickly at low doses, be active against latent bacteria, and have minimal effects on the liver so that they can be used in patients co-infected with HIV. In this study, the researchers investigated a chemical called OPC-67683.

What Did the Researchers Do and Find? The researchers identified a compound that inhibited the production of mycolic acid—an essential component of the cell wall of *M. tuberculosis*—and they tested its ability to kill the organism. They then tested in detail its ability to inhibit bacterial growth in dishes of antibiotic-sensitive and MDR *M. tuberculosis* and isolates from patients. OPC-67683 inhibited the growth of all these bugs at lower concentrations than the four antibiotics used in the standard TB treatment. It also killed bacteria hidden within human cells as well as or better than these drugs. Next, the researchers treated mice infected with *M. tuberculosis* with OPC-67683. They found that it reduced the number of bacteria in the lungs of both normal and immunocompromised mice at lower concentrations than the standard drugs. Furthermore, when combined with two of the standard drugs, it reduced the time taken to clear bacteria from the lungs by the standard drug regimen by two months. Finally, the researchers showed that OPC-67683 had no effects on the liver enzymes that metabolize antiretrovirals, and, conversely, that the activity of OPC-67683 was not affected by liver enzymes. Thus, this agent is unlikely to cause clinical problems or lose its efficacy in HIV patients who are receiving antiretroviral drugs.

What Do These Findings Mean? These results from laboratory and animal experiments suggest that OPC-67683 could possibly fulfill the criteria for a new anti-TB drug. OPC-67683 is active against MDR-TB. It is also active against intracellular TB, which the authors postulate could be a positive link with the effective treatment of latent TB, and it works quickly in animals when combined with existing anti-TB drugs. Importantly, it also disables *M. tuberculosis* in a unique way and does not appear to have any major effects on the liver that might stop it from being used in combination with antiretrovirals. All these preclinical characteristics now need to be checked in people—many drugs do well in preclinical studies but fail in patients. These clinical studies need to be expedited given the upsurge in TB, and, write the researchers, OPC-67683 needs to be tested in combination with both conventional drugs and other new drugs so that the best regimen of new drugs for the treatment of TB can be found as soon as possible.

Additional Information. Please access these Web sites via the online version of this summary at http://dx.doi.org/10.1371/journal.pmed.0030466.

- US National Institute of Allergy and Infectious Diseases patient fact sheet on tuberculosis
- US Centers for Disease Control and Prevention information on tuberculosis
- MedlinePlus encyclopedia entry on tuberculosis
- NHS Direct Online patient information on tuberculosis from the UK National Health Service
- World Health Organization information on the global elimination of tuberculosis
- Global Alliance for TB Drug Development information on why new TB drugs are needed

Synthesis and Antituberculosis Activity of a Novel Series of Optically Active 6-Nitro-2,3-dihydroimidazo[2,1-*b*]oxazoles

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In an effort to develop potent new antituberculosis agents that would be effective against both drug-susceptible and drug-resistant strains of *Mycobacterium tuberculosis*, we prepared a novel series of optically active 6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazoles substituted at the 2-position with various phenoxymethyl groups and a methyl group and investigated the *in vitro* and *in vivo* activity of these compounds. Several of these derivatives showed potent *in vitro* and *in vivo* activity, and compound **19** (OPC-67683) in particular displayed excellent *in vitro* activity against both drug-susceptible and drug-resistant strains of *M. tuberculosis* $H_{37}Rv$ (MIC = 0.006 μg/mL) and dose-dependent and significant *in vivo* efficacy at lower oral doses than rifampicin in mouse models infected with *M. tuberculosis* Kurono. The synthesis and structure−activity relationships of these new compounds are presented.

Introduction

Tuberculosis (TB),[a] an airborne lung infection, still remains a major public health problem worldwide. It is estimated that about 32% of the world population is infected with TB bacillus, and of those, approximately 8.9 million people develop active TB and 1.7 million die as a result annually according to 2004 figures.[1] Human immunodeficiency virus (HIV) infection has been a major contributing factor in the current resurgence of TB.[2,3] HIV-associated TB is widespread, especially in sub-Saharan Africa, and such an infectious process may further accelerate the resurgence of TB. Moreover, the recent emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* that are resistant to two major effective drugs, isonicotinic acid hydrazide (INH)[4] and rifampicin (RFP),[5] has further complicated the world situation.[6] The World Health Organization (WHO) has estimated that if the present conditions remain unchanged, more than 30 million lives will be claimed by TB between 2000 and 2020.[7] As for subsequent drug development, not a single new effective compound has been launched as an antituberculosis agent since the introduction of RFP in 1965, despite the great advances that have been made in drug development technologies.[3] Although many effective vaccine candidates have been developed, more potent vaccines will not become immediately available. The current therapy consists of an intensive phase with four drugs, INH, RFP, pyrazinamide (PZA),[8] and streptomycin (SM)[9] or ethambutol (EB),[10] administered for 2 months followed by a continuous phase with INH and RFP for 4 months.[11] Thus, there exists an urgent need for the development of potent new antituberculosis agents with low-toxicity profiles that are effective against both drug-susceptible and drug-resistant strains of *M. tuberculosis* and that are capable of shortening the current duration of therapy.[12]

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[a] Abbreviations: TB, tuberculosis; HIV, human immunodeficiency virus; MDR, multidrug-resistant; INH, isonicotinic acid hydrazide; RFP, rifampicin; PZA, pyrazinamide; SM, streptomycin; EB, ethambutol; MIC, minimum inhibitory concentration; CFU, colony forming unit; DMSO, dimethylsulfoxide.

Recognizing this serious situation, we initiated a program to screen for new antituberculosis agents. We synthesized and screened various compounds, including a number of dihydrophenazines,[13] indoles, and ureas.[14] One group of compounds on which we focused our attention was 6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazoles because of their inhibitory activity against mycolic acid biosynthesis,[14] which plays an important role in mycobacteria.[15] Nitroimidazoles, such as the nitroimidazole antibiotic metronidazole, are widely used for the treatment of anaerobic bacteria and protozoan infections, but they have had poor potency against *M. tuberculosis*.[16] In 1989, researchers at Ciba-Geigy reported the discovery of a bicyclic nitroimidazooxazole, **1** (CGI 17341)[17] (Figure 1), possessing favorable *in vitro* activity and *in vivo* efficacy. However, further investigation of **1** as an antituberculosis agent had to be discontinued due to the compound's mutagenicity.[18] Later, a research group at PathoGenesis Corporation developed a bicyclic nitroimidazopyran, **2** (PA-824),[19] that exhibited potent bactericidal activity against MDR *M. tuberculosis* and promising oral activity in animal infection models. We speculated that changing the substituents at the 2-position of 6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazoles, which have a structure similar to **1**, might enhance antituberculosis activity and eliminate mutagenicity. In our early experiments, however, no decrease in mutagenicity was achieved by introducing other alkyl substituents into the 2-position. After various experiments with different substituents, we succeeded in discovering a number of derivatives that did not exert mutagenicity from among compounds with heteroatoms in the side chains at the 2-position.[20] Therefore, to identify agents that display increased antituberculosis activity, we prepared a series of novel optically active 6-nitro-2,3-dihydroimidazo[2,1-*b*]-oxazoles having various phenoxymethyl groups and a methyl group at the 2-position. As a result of extensive evaluation, we found a potent, orally active compound that is a promising candidate for the treatment of tuberculosis. We describe herein the synthesis and biological activity of these novel agents.

Chemistry

The first objective of this investigation was to immediately synthesize a variety of (*R*)-form derivatives and evaluate their

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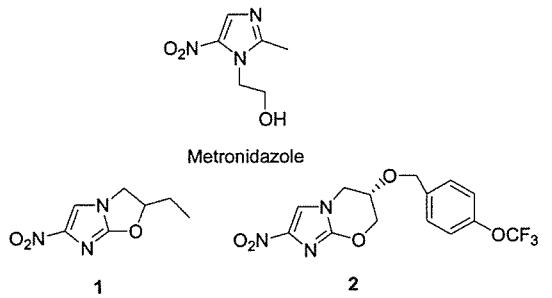
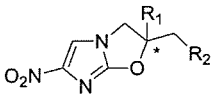


Figure 1. Metronidazole and bicyclic nitroimidazole derivatives **1** and **2**.

Table 1. *In Vitro* MIC Values of **3a**–**g**



compd	R_1	R_2	configuration	MIC (μg/mL)[a]
3a	H	OPh	racemic	0.78
3b	H	OCH_2Ph	racemic	3.13
3c	H	$O(CH_2)_2Ph$	racemic	1.56
3d	H	$OCH_2CH{=}CHPh$	racemic	12.5
3e	Me	OPh	racemic	0.1
3f	Me	OPh	(*R*)	0.05
3g	Me	OPh	(*S*)	3.13

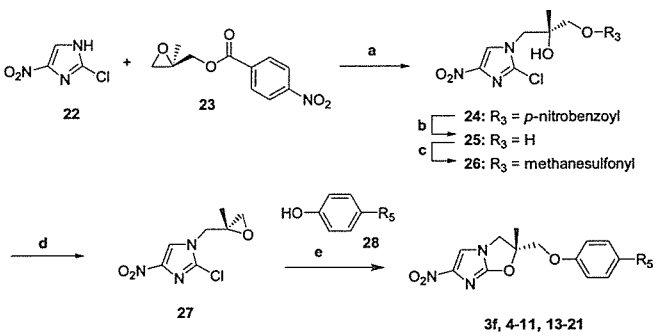
[a] MIC against *M. tuberculosis* $H_{37}Rv$. MIC of RFP = 0.1–0.39 μg/mL.

in vitro and *in vivo* activity. Second, through screening, we intended to identify a potent agent having no mutagenicity as a candidate for the treatment of tuberculosis.

We first synthesized the four racemic compounds **3a**–**d** essentially according to previously reported methods (Table 1).[17,21] Their minimum inhibitory concentration (MIC) values against *M. tuberculosis* $H_{37}Rv$[22] were, respectively, 0.78, 3.13, 1.56, and 12.5 μg/mL, with **3a**, which has a phenoxymethyl group at the 2-position, providing the best result. We then prepared compound **3e**, which has a methyl group at the 2-position of **3a**. Compared with **3a**, **3e** showed increased inhibitory activity (MIC = 0.1 μg/mL). Furthermore, comparison of (*R*)-form **3f** (MIC = 0.05 μg/mL) with (*S*)-form **3g** (MIC = 3.13 μg/mL) showed the (*R*)-form to be the more active form. The synthesis method for these two optically active compounds will be described later. Accordingly, we decided to develop (*R*)-derivatives with various substituted-phenoxymethyl groups and a methyl group at the 2-position to obtain a more potent compound.

Our synthesis strategy for preparation of the optically active 6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazoles with substituted-phenoxymethyl groups **3f** and **4**–**21** ((*R*)-form) involved the utilization of the key intermediate (*R*)-form **27**, an optically active epoxide easily derived from 2-chloro-4-nitro-1*H*-imidazole (**22**)[23] and (*R*)-2-methyl-2,3-epoxypropyl 4-nitrobenzoate (**23**)[24] (Scheme 1). Namely, compound **22** was reacted with the epoxide **23** in the presence of triethylamine in ethyl acetate to afford **24**, followed by de-esterification with methanol and a catalytic amount of potassium carbonate to give the diol **25**. The thus obtained diol was allowed to react with methanesulfonyl chloride in pyridine to afford the mesylate **26**, which was easily converted into the (*R*)-form epoxide **27** with 1,8-diazabicyclo[5.4.0]-7-undecene in ethyl acetate. Finally, the target compounds were synthesized by coupling **27** with various phenol compounds **28**, followed by ring closure in the presence of sodium hydride in *N*,*N*-dimethylformamide.

Scheme 1[a]



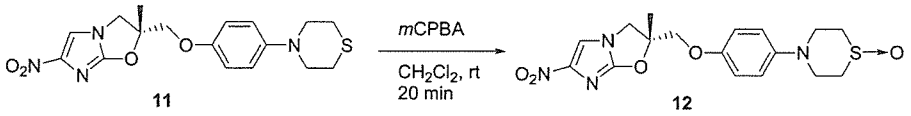
[a] Reagents: (a) Et_3N, AcOEt, 60–65 °C, 6 h; (b) K_2CO_3, MeOH, rt, 2 h; (c) MsCl, pyridine, <15 °C, 2 h; (d) DBU, AcOEt, rt, 2 h; (e) **28**, NaH, DMF, 50 °C, 2 h.

The (*S*)-form **3g** was similarly prepared by using the (*S*)-form epoxide **29** instead of **27** essentially according to the same method. Compound **12** was synthesized by oxidation of **11** with *m*-chloroperbenzoic acid in dichloromethane (Scheme 2). Among the phenol compounds **28**, 4-(piperidin-1-yl)phenol for **9**, 4-(morpholin-4-yl)phenol for **10**, and 4-(thiomorpholin-4-yl)-phenol for **11** were obtained according to the previously reported methods.[25] The synthesis method for the phenol compounds **28a**–**i** for preparing **13**–**21** was as follows: 2-(4-bromophenoxy)tetrahydropyran (**30**)[26] was reacted with various 4-phenoxypiperidine derivatives **31**[27] by the Buchwald palladium-catalyzed amination method[28] to afford **32**. The thus-obtained **32** was deprotected with pyridinium *p*-toluenesulfonate in ethanol to give the desired phenols **28a**–**i** (Scheme 3). All synthesized (*R*)-form compounds are displayed in Tables 2 and 3, and each compound was chemically characterized by melting point and nuclear magnetic resonance (^1H NMR), as well as by elemental microanalysis.

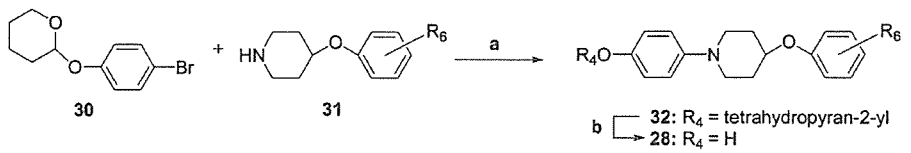
Results and Discussion

All compounds **3f** and **4**–**21** prepared in this investigation were tested for *in vitro* antituberculosis activity against both drug-susceptible and drug-resistant strains of *M. tuberculosis* $H_{37}Rv$[22] and for short-term *in vivo* efficacy at an oral dose of 50 mg/kg for 10 days in mice infected with *M. tuberculosis* Kurono[11] as the primary screening model. The results are summarized in Tables 2 and 3. Among the compounds **3f** and **4**–**8** (Table 2), **3f** (H), **4** (Cl), and **5** (Me) showed high *in vitro* activity and significant *in vivo* efficacy. However, the *in vivo* efficacy of **6** (MeO) was found to be inferior to that of **3f** despite its high *in vitro* activity. Although **7** (CF_3) and **8** (OCF_3) showed only moderate MIC values, they exhibited more potent *in vivo* efficacy than **3f**. Compounds **9**–**12** (Table 2), designed to improve bioavailability by the introduction of hydrophilic substituents into the 4-position of the benzene ring of **3f**, also had moderate MIC values, except for **12**, but unexpectedly their *in vivo* efficacy was generally poor in comparison with **3f**. Because **9** (piperidino) showed the most potent *in vivo* efficacy, (1.9 log CFU reduction in mouse lung) among these four compounds having hydrophilic substituents, we prepared compounds **13**–**21** (Table 3) by introducing lipophilic phenoxy groups to the 4-position of the piperidine ring of **9** to search for more potent agents. Among compounds **13**–**17**, **14** (*p*-Cl) exhibited high *in vitro* activity and **13** (H), **14**, and **15** (*p*-F) showed increased *in vivo* efficacy in comparison with **9**. However, **16** (*p*-Me) and **17** (*p*-MeO) did not show efficacy in *in vivo* screening, contrary to our expectations. In particular, **18** (*p*-CF_3) and **19** (*p*-OCF_3) both showed similar excellent *in*

Scheme 2

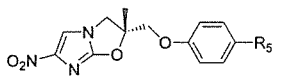


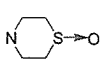
Scheme 3[a]



[a] Reagents: (a) $Pd(OAc)_2$, *rac*-BINAP, Cs_2CO_3, toluene, reflux, 30 min; (b) pyridinium *p*-toluenesulfonate, EtOH, 70 °C, 24 h.

Table 2. *In Vitro* and *in Vivo* Activity of Synthesized Compounds **3f** and **4**−**12**



Compd	R_5	MIC (μg/mL) against *M. tuberculosis* strains			log CFU reduction[b]
		$H_{37}Rv$[a]	$H_{37}Rv$ INH-resistant	$H_{37}Rv$ RFP-resistant	
3f	H	0.05	0.05	0.05	2.0
4	Cl	0.024	0.012	0.006	> 3.1
5	Me	0.012	0.024	0.012	2.9
6	MeO	0.05	0.1	0.05	0.72
7	CF_3	0.2	0.2	0.1	> 4.4
8	OCF_3	0.2	0.39	0.2	> 3.6
9		0.78	0.39	0.39	1.9
10		0.78	0.78	0.39	1.3
11		0.78	0.39	0.2	0.0
12		6.25	6.25	6.25	ND[c]

[a] MIC of RFP against *M. tuberculosis* $H_{37}Rv$ = 0.1−0.39 μg/mL. [b] log CFU reduction in mouse lung relative to untreated controls by once-daily oral administration at 50 mg/kg for 10 days (n = 2) starting on the day after intravenous infection with 10^4 CFU of *M. tuberculosis* Kurono. [c] ND = not determined.

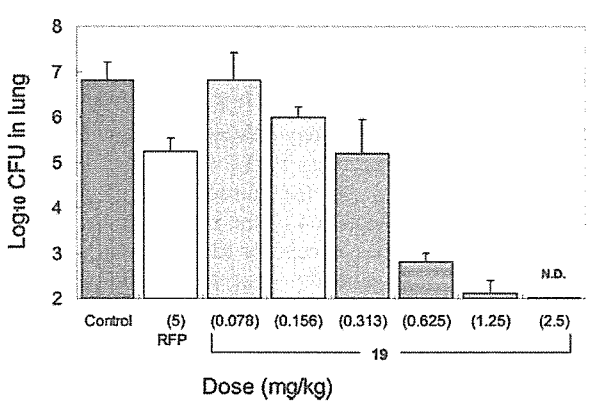


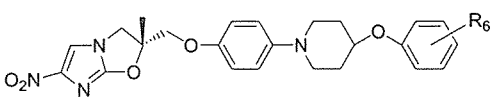
Figure 2. *In vivo* efficacy of compound **19** against *M. tuberculosis* Kurono. Mice were orally dosed once daily for 28 days (n = 6) starting on the day after intravenous infection with 10^4 CFU of mycobacteria.

vitro activity, but **19** was superior to **18** regarding *in vivo* potency (>3.8 log CFU reduction). The excellent *in vitro* activity of **19** was mirrored by its significant *in vivo* efficacy in the mouse acute model. Although **20** (*o*-OCF_3) and **21** (*m*-OCF_3), synthesized by converting the positions of a trifluoromethoxy group of **19** into *ortho* or *meta*, were found to have less potent *in vitro* activity than **19**; the *in vivo* efficacy of **21** was found to be similar to that of **19**.

Next, the compounds **8**, which showed potent *in vivo* efficacy, and **19**, which demonstrated the highest *in vitro* activity among all of the synthesized compounds, were then evaluated *in vivo* at oral doses of 0.5 and 10 mg/kg for 10 days (Table 4). In this *in vivo* test, RFP at 5 mg/kg was used as a reference compound. Compounds **8** and **19** both showed a significant decrease in bacterial load in this evaluation. The oral activity of **8** at a dose of 0.5 mg/kg was similar to that of RFP at 5 mg/kg, and even more notably, oral administration of **19** at a dose of 0.5 mg/kg produced a much better result than RFP at 5 mg/kg. Consequently, based on these evaluation results, compound **19** was selected for further scrutiny.

Finally, **19** was tested for *in vivo* efficacy at lower oral doses of 0.078−2.5 mg/kg once daily for 28 days in mice infected with *M. tuberculosis* Kurono (Figure 2) as a model system. The results for RFP at a dose of 5 mg/kg as a reference drug are

Table 3. *In Vitro* and *in Vivo* Activity of Synthesized Compounds **13**–**21**



compd	R_6	MIC (μg/mL) against *M. tuberculosis* strains: $H_{37}Rv$[a]	$H_{37}Rv$ INH-resistant	$H_{37}Rv$ RFP-resistant	log CFU reduction[b]
13	H	0.39	0.39	0.2	2.8
14	*p*-Cl	0.05	0.05	0.024	2.2
15	*p*-F	0.39	0.39	0.2	2.2
16	*p*-Me	0.78	0.39	0.39	0.6
17	*p*-MeO	0.39	0.39	0.2	0.1
18	*p*-CF_3	0.012	0.012	0.006	2.2
19	*p*-OCF_3	0.006	0.006	0.006	>3.8
20	*o*-OCF_3	0.39	0.39	0.2	3.0
21	*m*-OCF_3	0.024	0.024	0.024	>4.4

[a] MIC of RFP against *M. tuberculosis* $H_{37}Rv$ = 0.1–0.39 μg/mL. [b] log CFU reduction in mouse lung relative to untreated controls by once-daily oral administration at 50 mg/kg for 10 days (n = 2) starting on the day after intravenous infection with 10^4 CFU of *M. tuberculosis* Kurono.

Table 4. *In Vivo* Efficacy of Compounds **8** and **19** as Compared with RFP

compd	**19**		**8**		RFP
dose (mg/kg)	0.5	10	0.5	10	5
log CFU reduction[a]	2.5	>4.4	0.4	3.0	0.5

[a] 10-day treatment of mouse model infection with *M. tuberculosis* Kurono similar to Tables 1 and 2.

also presented. Compound **19** showed a dose-dependent and significant decrease in mouse pulmonary *M. tuberculosis* bacterial counts. In particular, the efficacy of **19** at 0.313 mg/kg was comparable to that of RFP at 5 mg/kg. This potent compound **19** had none of the mutagenicity previously associated with **1**.[20] Therefore, based on the screening results, we selected compound **19** as an orally active candidate for the treatment of tuberculosis.

Conclusions

Screening of this novel series of (*R*)-form optically active 6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole derivatives for *in vitro* antituberculosis activity and *in vivo* oral efficacy indicated that compounds with substituted phenoxymethyl groups and a methyl group at the 2-position are a new class of agents endowed with highly potent antituberculosis activity. Due to its excellent *in vitro* antituberculosis activity against both drug-susceptible and drug-resistant strains of *M. tuberculosis* $H_{37}Rv$ and its potent *in vivo* efficacy in mice infected with *M. tuberculosis* Kurono as a model system, compound **19** was concluded to be a promising orally active candidate for the treatment of tuberculosis. Most notably, compound **19** at an oral dose of 0.313 mg/kg for 28 days showed *in vivo* efficacy comparable to that of RFP at 5 mg/kg. Still more detailed biological data will be presented in a separate paper.[20] Compound **19** (OPC-67683)[20] is now under intensive development.

Experimental Section

General Methods. Reagents were used as supplied unless otherwise noted. All melting points were determined on a Yanaco MP-500D apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker DPX250 instrument operating at 250 MHz. Chemical shifts are shown in parts per million (ppm) on the δ scale downfield relative to tetramethylsilane as an internal standard, and coupling constants are shown in hertz (Hz). Optical rotations were measured on a JASCO DPI-370 digital polarimeter. Satisfactory spectral data were obtained for all of the new compounds. Satisfactory elemental analyses (±0.4%) were obtained for all crystalline derivatives. Chromatographic separations were performed on silica gel columns by gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography.

Racemic compounds **3b**–**e** were essentially prepared according to the previously reported methods.[17,21] Compound **3a** has been previously reported.[17]

2-Benzyloxymethyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (3b). Mp 125–126 °C. ^{1}H NMR ($CDCl_3$) δ 3.76 (1H, dd, J = 3.5 Hz, 11.2 Hz), 3.87 (1H, dd, J = 4.1 Hz, 11.2 Hz), 4.23–4.34 (2H, m), 4.59 (2H, s), 5.34–5.43 (1H, m), 7.23–7.41 (5H, m), 7.52 (1H, s). MS (DI) *m/z* 276 (M^+ + 1). Anal. ($C_{13}H_{13}N_3O_4$) C, H, N.

6-Nitro-2-phenethyloxymethyl-2,3-dihydroimidazo[2,1-*b*]oxazole (3c). Mp 115–116 °C. ^{1}H NMR ($CDCl_3$) δ 2.84 (2H, t, J = 6.6 Hz), 3.64–3.86 (4H, m), 4.09 (1H, dd, J = 6.2 Hz, 10.0 Hz), 4.21 (1H, dd, J = 8.6 Hz, 10.0 Hz), 5.25–5.41 (1H, m), 7.07–7.32 (5H, m), 7.46 (1H, s). MS (DI) *m/z* 289 (M^+). Anal. ($C_{14}H_{15}N_3O_4$) C, H, N.

2-Cinnamyloxymethyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (3d). Mp 145–147 °C. ^{1}H NMR ($CDCl_3$) δ 3.80 (1H, dd, J = 3.6 Hz, 11.3 Hz), 3.90 (1H, dd, J = 4.0 Hz, 11.3 Hz), 4.20–4.34 (4H, m), 5.34–5.50 (1H, m), 6.22 (1H, ddd, J = 6.2 Hz, 12.4 Hz, 16.0 Hz), 6.57 (1H, d, J = 16.0 Hz), 7.20–7.39 (5H, m), 7.54 (1H, s). MS (DI) *m/z* 302 (M^+). Anal. ($C_{15}H_{15}N_3O_4$) C, H, N.

2-Methyl-6-nitro-2-phenoxymethyl-2,3-dihydroimidazo[2,1-*b*]oxazole (3e). Mp 117–119 °C. ^{1}H NMR ($CDCl_3$) δ 1.79 (3H, s), 4.03 (1H, d, J = 10.2 Hz), 4.09 (1H, d, J = 10.2 Hz), 4.24 (1H, d, J = 10.1 Hz), 4.50 (1H, d, J = 10.1 Hz), 6.84 (2H, dd, J = 2.0 Hz, 8.6 Hz), 7.01 (1H, t, J = 7.4 Hz), 7.20–7.31 (2H, m), 7.56 (1H, s). MS (DI) *m/z* 275 (M^+). Anal. ($C_{13}H_{13}N_3O_4$) C, H, N.

(*R*)-2-Chloro-1-[2-hydroxy-2-methyl-3-(4-nitrobenzoyloxy)]-propyl-4-nitroimidazole (24). A solution of 2-chloro-4-nitro-1*H*-imidazole (**22**)[23] (3 g, 20.34 mmol), (*R*)-form epoxide **23**[24] (5.31 g, 22.37 mmol), and triethylamine (0.57 mL, 4.07 mmol) in ethyl acetate (10 mL) was heated at 60–65 °C for 6 h. The reaction mixture was allowed to cool to room temperature and concentrated under reduced pressure. To the residue was added ethyl acetate (3 mL) and toluene (30 mL). The resulting precipitates were collected by filtration and recrystallized from ethyl acetate–isopropylether to give **24** (6.82 g, 87%) as colorless needles. Mp 122–123 °C. ^{1}H NMR (DMSO-d_6) δ 1.23 (3H, s), 4.11–4.33 (4H, m), 5.61 (1H, s), 8.25 (2H, d, J = 8.9 Hz), 8.31–8.45 (3H, m). $[\alpha]_D^{26}$ 54.0° (*c* 1.04, CH_3CN). MS (DI) *m/z* 384 (M^+). Anal. ($C_{14}H_{13}ClN_4O_7$) C, H, N.

(*R*)-2-Chloro-1-(2,3-dihydroxy-2-methyl)propyl-4-nitroimidazole (25). To a solution of **24** (6.80 g, 17.67 mmol) in methanol (68 mL) was added potassium carbonate (122 mg, 0.88 mmol). After the solution was stirred at room temperature for 2 h, 6 M hydrochloric acid (0.3 mL) and magnesium sulfate (3 g) were added at 0 °C, and the resulting mixture was stirred for 1 h. The insoluble materials were filtered off through Celite, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (dichloromethane/methanol = 20/1) and recrystallized from ethyl acetate–isopropylether to give **25** (4.09 g, 97%) as colorless needles. Mp 110–111 °C. ^{1}H NMR (DMSO-d_6) δ 1.01 (3H, s), 3.25 (2H, d, J = 5.3 Hz), 4.05 (2H, s), 5.01 (1H, s), 5.11 (1H, t, J = 5.4 Hz), 8.32(1H, s). $[\alpha]_D^{27}$ 17.4° (*c* 1.03, DMSO). MS (DI) *m/z* 235 (M^+). Anal. ($C_7H_{10}ClN_3O_4$) C, H, N.

(*R*)-2-Chloro-1-(2-methyl-2,3-epoxypropyl)-4-nitroimidazole (27). To a solution of **25** (10 g, 42.44 mmol) in pyridine (20 mL) was added methanesulfonyl chloride (7.29 g, 63.66 mmol) at below 15 °C dropwise over 30 min. After the solution was stirred for 2 h, 6 M hydrochloric acid (63 mL) was added to the reaction mixture at below 30 °C. The resulting mixture was extracted with ethyl acetate (75 mL × 2), and the combined organic layer was washed with brine, dried over magnesium sulfate, and filtered. The

filtrate was concentrated under reduced pressure, and to the residue was added toluene (75 mL). The resulting precipitates were collected by filtration to afford crude **26**. To a solution of this crude **26** in ethyl acetate (100 mL) was added 1,8-diazabicyclo[5.4.0]-7-undecene (7.10 g, 46.68 mmol), and the mixture was stirred at room temperature for 2 h. The reaction mixture was washed with brine, dried over magnesium sulfate, and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (ethy acetate/hexane = 1/1) to give the (*R*)-form epoxide **27** (6.93 g, 75%) as colorless needles. Mp 72−73 °C. ^{1}H NMR ($CDCl_3$) δ 1.38 (3H, s), 2.62 (1H, d, J = 4.0 Hz), 2.78 (1H, d, J = 4.0 Hz), 4.00 (1H, d, J = 14.9 Hz), 4.38 (1H, d, J = 14.9 Hz), 7.87 (1H, s). $[\alpha]_D^{26}$ 31.1° (*c* 2.02, $CHCl_3$). MS (DI) *m/z* 217 (M^+). Anal. ($C_7H_8ClN_3O_3$) C, H, N.

(*S*)-2-Chloro-1-(2-methyl-2,3-epoxypropyl)-4-nitroimidazole (29). This compound was obtained by the same procedure as described for **27** from 2-chloro-4-nitro-1*H*-imidazole (**22**) and (*S*)-2-methyl-2,3-epoxypropyl 4-nitrobenzoate.[24] Mp 72−73 °C. ^{1}H NMR ($CDCl_3$) δ 1.39 (3H, s), 2.63 (1H, d, J = 4.0 Hz), 2.79 (1H, d, J = 4.0 Hz), 4.00 (1H, d, J = 14.9 Hz), 4.38 (1H, d, J = 14.9 Hz), 7.88 (1H, s). $[\alpha]_D^{27}$ −29.2° (*c* 1.18, $CHCl_3$). MS (DI) *m/z* 217 (M^+). Anal. ($C_7H_8ClN_3O_3$) C, H, N.

1-[4-(Tetrahydropyran-2-yloxy)phenyl]-4-(4-trifluoromethoxyphenoxy)piperidine (32g). A mixture of 2-(4-bromophenoxy)-tetrahydropyran (**30**)[26] (30 g, 116.67 mmol) and 4-(4-trifluoromethoxyphenoxy)piperidine (**31g**)[27] (30.30 g, 115.60 mmol) in the presence of palladium acetate (1 g, 4.64 mmol), *rac*-2,2′-bis-(diphenylphosphino)-1,1′-binaphthyl (4.30 g, 6.96 mmol), and cesium carbonate (49 g, 150.39 mmol) in toluene (300 mL) was refluxed under a nitrogen atmosphere for 30 min. The reaction mixture was allowed to cool to room temperature, and ethyl acetate (300 mL) and water (200 mL) were added. The thus-obtained mixture was filtered through Celite. The organic layer was separated, washed with brine, dried over magnesium sulfate, and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (ethy acetate/hexane = 1/20) to give **32g** (32.60 g, 64%) as a yellow crystalline powder. ^{1}H NMR ($CDCl_3$) δ 1.55−1.75 (3H, m), 1.81−2.20 (7H, m), 2.95−3.04 (2H, m), 3.38−3.42 (2H, m), 3.55−3.66 (1H, m), 3.87−3.99 (1H, m), 3.56−4.45 (1H, m), 5.29−5.32 (1H, m), 6.89−7.01 (6H, m), 7.11−7.16 (2H, m).

4-[4-(4-Trifluoromethoxyphenoxy)piperidin-1-yl]phenol (28g). A mixture of **32g** (30.10 g, 68.81 mmol) and pyridinium *p*-toluenesulfonate (5.20 g, 20.69 mmol) in ethanol (450 mL) was heated at 70 °C for 24 h. The reaction mixture was allowed to cool to room temperature and concentrated under reduced pressure. Saturated sodium hydrogen carbonate aqueous solution (100 mL) was added to the residue, which was extracted with dichloromethane (200 mL). The organic layer was washed with brine, dried over magnesium sulfate, and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (dichloromethane/ethyl acetate = 10/1) to give **28g** (22.90 g, 94%) as a pale yellow crystalline powder. ^{1}H NMR ($CDCl_3$) δ 1.88−2.02 (2H, m), 2.06−2.16 (2H, m), 2.92−3.02 (2H, m), 3.30−3.39 (2H, m), 4.36−4.44 (1H, m), 4.74 (1H, s), 6.71−6.78 (2H, m), 6.85−6.94 (4H, m), 7.10−7.16 (2H, m).

Other phenol derivatives **28a−f** and **28h,i** were synthesized by the same procedure as described for **28g**. Compounds **28a−i** were immediately used for the next reaction.

4-(4-Phenoxypiperidin-1-yl)phenol (28a). ^{1}H NMR ($CDCl_3$) δ 1.89−2.03 (2H, m), 2.04−2.18 (2H, m), 2.92−3.02 (2H, m), 3.31−3.41 (2H, m), 4.39−4.49 (1H, m), 4.92 (1H, s), 6.70−6.78 (2H, m), 6.84−6.98 (5H, m), 7.24−7.33 (2H, m).

4-[4-(4-Chlorophenoxy)piperidin-1-yl]phenol (28b). ^{1}H NMR ($CDCl_3$) δ 1.87−2.01 (2H, m), 2.04−2.16 (2H, m), 2.91−3.02 (2H, m), 3.29−3.39 (2H, m), 4.34−4.44 (1H, m), 4.85 (1H, s), 6.71−6.78 (2H, m), 6.82−6.92 (4H, m), 7.20−7.26 (2H, m).

4-[4-(4-Fluorophenoxy)piperidin-1-yl]phenol (28c). ^{1}H NMR ($CDCl_3$) δ 1.86−2.00 (2H, m), 2.04−2.16 (2H, m), 2.90−3.00 (2H, m), 3.30−3.40 (2H, m), 4.29−4.39 (1H, m), 4.72 (1H, s), 6.71−6.78 (2H, m), 6.83−7.01 (6H, m).

4-[4-(4-Methylphenoxy)piperidin-1-yl]phenol (28d). ^{1}H NMR ($CDCl_3$) δ 1.87−2.01 (2H, m), 2.04−2.16 (2H, m), 2.29 (3H, s), 2.90−3.00 (2H, m), 3.30−3.40 (2H, m), 4.33−4.43 (1H, m), 4.85 (1H, s), 6.71−6.78 (2H, m), 6.80−6.92 (4H, m), 7.06−7.10 (2H, m).

4-[4-(4-Methoxyphenoxy)piperidin-1-yl]phenol (28e). ^{1}H NMR ($CDCl_3$) δ 1.86−2.00 (2H, m), 2.05−2.13 (2H, m), 2.88−2.99 (2H, m), 3.31−3.41 (2H, m), 3.77 (3H, s), 4.25−4.35 (1H, m), 4.72 (1H, s), 6.72−6.77 (2H, m), 6.80−6.92 (6H, m).

4-[4-(4-Trifluoromethylphenoxy)piperidin-1-yl]phenol (28f). ^{1}H NMR ($CDCl_3$) δ 1.90−2.05 (2H, m), 2.08−2.20 (2H, m), 2.94−3.05 (2H, m), 3.30−3.40 (2H, m), 4.46−4.56 (1H, m), 4.64 (1H, s), 6.72−6.80 (2H, m), 6.86−6.93 (2H, m), 6.96−7.00 (2H, m), 7.52−7.56 (2H, m).

4-[4-(2-Trifluoromethoxyphenoxy)piperidin-1-yl]phenol (28h). ^{1}H NMR ($CDCl_3$) δ 1.91−2.16 (4H, m), 2.91−3.07 (2H, m), 3.25−3.40 (2H, m), 4.40−4.53 (1H, m), 4.70 (1H, s), 6.76 (2H, dd, J = 2.3 Hz, 6.7 Hz), 6.81−7.05 (4H, m), 7.12−7.28 (2H, m).

4-[4-(3-Trifluoromethoxyphenoxy)piperidin-1-yl]phenol (28i). ^{1}H NMR ($CDCl_3$) δ 1.89−2.18 (4H, m), 2.94−3.06 (2H, m), 3.27−3.41 (2H, m), 4.35−4.51 (1H, m), 4.71 (1H, s), 6.71−6.96 (7H, m), 7.25−7.35 (1H, m).

(*R*)-2-Methyl-6-nitro-2-{4-[4-(4-trifluoromethoxyphenoxy)piperidin-1-yl]phenoxymethyl}-2,3-dihydroimidazo[2,1-*b*]oxazole (19). To a mixture of **27** (127.56 g, 586.56 mmol) and 4-[4-(4-trifluoromethoxyphenoxy)piperidin-1-yl]phenol (**28g**) (165.70 g, 468.95 mmol) in *N*,*N*-dimethylformamide (1600 mL) was added 60% sodium hydride (22.51 g, 562.74 mmol) at 0 °C portionwise. After the mixture was stirred at 50 °C for 2 h under a nitrogen atmosphere, the reaction mixture was cooled in an ice bath and carefully quenched with ethyl acetate (230 mL) and ice water (50 mL). The thus-obtained mixture was poured into water (3000 mL) and stirred for 30 min. The resulting precipitates were collected by filtration, washed with water, and dried at 60 °C overnight. This crude product was purified by silica gel column chromatography using a dichloromethane and ethyl acetate mixture (5/1) as solvent. The appropriate fractions were combined and evaporated under reduced pressure. The residue was recrystallized from ethyl acetate (1300 mL)−isopropyl alcohol (150 mL) to afford **19** (119.11 g, 48%) as a pale yellow crystalline powder. Mp 195−196 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 1.87−2.16 (4H, m), 2.95−3.05 (2H, m), 3.32−3.41 (2H, m), 4.02 (1H, d, J = 10.2 Hz), 4.04 (1H, d, J = 10.2 Hz), 4.18 (1H, J = 10.2 Hz), 4.36−4.45 (1H, m), 4.49 (1H, d, J = 10.2 Hz), 6.76 (2H, d, J = 6.7 Hz), 6.87−6.94 (4H, m), 7.14 (2H, d, J = 8.6 Hz), 7.55 (1H, s). $[\alpha]_D^{28}$ −9.9° (*c* 1.01, $CHCl_3$). MS (DI) *m/z* 535 ($M^+ + 1$). Anal. ($C_{25}H_{25}F_3N_4O_6$) C, H, N.

Compounds **3f, 4−11, 13−18, 20**, and **21** were prepared by the same procedure as described for **19**.

(*R*)-2-Methyl-6-nitro-2-phenoxymethyl-2,3-dihydroimidazo-[2,1-*b*]oxazole (3f). Mp 151−153 °C. ^{1}H NMR ($CDCl_3$) δ 1.79 (3H, s), 4.03 (1H, d, J = 10.2 Hz), 4.09 (1H, d, J = 10.2 Hz), 4.24 (1H, d, J = 10.1 Hz), 4.50 (1H, d, J = 10.1 Hz), 6.84 (2H, dd, J = 1.8 Hz, 8.5 Hz), 7.01 (1H, t, J = 7.2 Hz), 7.21−7.31 (2H, m), 7.55 (1H, s). MS (DI) *m/z* 275 (M^+). Anal. ($C_{13}H_{13}N_3O_4$) C, H, N.

(*R*)-2-(4-Chlorophenoxymethyl)-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (4). Mp 185−187 °C. ^{1}H NMR ($CDCl_3$) δ 1.78 (3H, s), 4.04 (1H, d, J = 3.2 Hz), 4.08 (1H, d, J = 3.2 Hz), 4.21 (1H, d, J = 10.1 Hz), 4.49 (1H, d, J = 10.1 Hz), 6.78 (2H, d, J = 9.0 Hz), 7.19−7.29 (2H, m), 7.56 (1H, s). MS (DI) *m/z* 309 (M^+). Anal. ($C_{13}H_{12}ClN_3O_4$) C, H, N.

(*R*)-2-Methyl-2-(4-methylphenoxymethyl)-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (5). Mp 177−179 °C. ^{1}H NMR ($CDCl_3$) δ 1.78 (3H, s), 2.28 (3H, s), 4.02 (1H, d, J = 7.2 Hz), 4.06 (1H, d, J = 7.2 Hz), 4.20 (1H, d, J = 10.1 Hz), 4.49 (1H, d, J = 10.1 Hz), 6.74 (2H, d, J = 8.3 Hz), 7.08 (2H, d, J = 8.3 Hz), 7.55 (1H, s). MS (DI) *m/z* 289 (M^+). Anal. ($C_{14}H_{15}N_3O_4$) C, H, N.

(*R*)-2-(4-Methoxyphenoxymethyl)-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (6). Mp 179−180 °C. ^{1}H NMR ($CDCl_3$)

δ 1.77 (3H, s), 3.76 (3H, s), 4.02 (1H, d, J = 2.6 Hz), 4.06 (1H, d, J = 2.6 Hz), 4.17 (1H, d, J = 10.2 Hz), 4.50 (1H, d, J = 10.2 Hz), 6.71−6.86 (4H, m), 7.55 (1H, s). MS (DI) *m/z* 305 (M^+). Anal. ($C_{14}H_{15}N_3O_5$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-(4-trifluoromethylphenoxymethyl)-2,3-dihydroimidazo[2,1-*b*]oxazole (7). Mp 188−190 °C. ^{1}H NMR ($CDCl_3$) δ 1.81 (3H, s), 4.08 (1H, d, J = 10.3 Hz), 4.18 (1H, d, J = 10.3 Hz), 4.29 (1H, d, J = 10.3 Hz), 4.50 (1H, d, J = 10.3 Hz), 6.93 (2H, d, J = 8.7 Hz), 7.50−7.59 (3H, m). MS (DI) *m/z* 343 (M^+). Anal. ($C_{14}H_{12}F_3N_3O_4$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-(4-trifluoromethoxyphenoxymethyl)-2,3-dihydroimidazo[2,1-*b*]oxazole (8). Mp 176−178 °C. ^{1}H NMR ($CDCl_3$) δ 1.79 (3H, s), 4.06 (1H, d, J = 6.8 Hz), 4.10 (1H, d, J = 6.8 Hz), 4.23 (1H, d, J = 10.1 Hz), 4.49 (1H, d, J = 10.1 Hz), 6.84 (2H, d, J = 9.0 Hz), 7.13 (2H, d, J = 9.0 Hz), 7.56 (1H, s). MS (DI) *m/z* 359 (M^+). Anal. ($C_{14}H_{12}F_3N_3O_5$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-[4-(piperidin-1-yl)phenoxymethyl]-2,3-dihydroimidazo[2,1-*b*]oxazole (9). Mp 217−219 °C. ^{1}H NMR ($CDCl_3$) δ 1.45−1.57 (5H, m), 1.61−1.78 (4H, m), 2.94−3.08 (4H, m), 4.00 (1H, d, J = 7.4 Hz), 4.04 (1H, d, J = 7.4 Hz), 4.17 (1H, d, J = 10.1 Hz), 4.49 (1H, d, J = 10.1 Hz), 6.75 (2H, d, J = 6.8 Hz), 6.89 (2H, d, J = 6.8 Hz), 7.54 (1H, s). MS (DI) *m/z* 358 (M^+). Anal. ($C_{18}H_{22}N_4O_4$) C, H, N.

(*R*)-2-Methyl-2-[4-(morpholin-4-yl)phenoxymethyl]-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (10). Mp 233−235 °C. ^{1}H NMR (DMSO-d_6) δ 1.67 (3H, s), 2.92−3.00 (4H, m), 3.61−3.71 (4H, m), 4.08−4.22 (3H, m), 4.36 (1H, d, J = 10.9 Hz), 6.80 (2H, d, J = 6.8 Hz), 6.88 (2H, d, J = 6.8 Hz), 8.15 (1H, s). MS (DI) *m/z* 360 (M^+). Anal. ($C_{17}H_{20}N_4O_5$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-[4-(thiomorpholin-4-yl)phenoxymethyl]-2,3-dihydroimidazo[2,1-*b*]oxazole (11). Mp 227−229 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 2.69−2.80 (4H, m), 3.31−3.71 (4H, m), 4.01 (1H, d, J = 5.5 Hz), 4.05 (1H, d, J = 5.5 Hz), 4.18 (1H, d, J = 10.1 Hz), 4.49 (1H, d, J = 10.1 Hz), 6.77 (2H, d, J = 6.7 Hz), 6.86 (2H, d, J = 6.7 Hz), 7.55 (1H, s). MS (DI) *m/z* 376 (M^+). Anal. ($C_{17}H_{20}N_4O_4S$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-[4-(4-phenoxypiperidin-1-yl)phenoxymethyl]-2,3-dihydroimidazo[2,1-*b*]oxazole (13). Mp 195−197 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 1.86−2.18 (4H, m), 2.92−3.08 (2H, m), 3.31−3.47 (2H, m), 4.01 (1H, d, J = 5.8 Hz), 4.05 (1H, d, J = 5.8 Hz), 4.18 (1H, d, J = 10.2 Hz), 4.37−4.55 (2H, m), 6.78 (2H, dd, J = 2.2 Hz, 6.8 Hz), 6.84−7.00 (5H, m), 7.20−7.33 (2H, m), 7.55 (1H, s). MS (DI) *m/z* 450 (M^+). Anal. ($C_{24}H_{26}N_4O_5$) C, H, N.

(*R*)-2-{4-[4-(4-Chlorophenoxy)piperidin-1-yl]phenoxymethyl}-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (14). Mp 183−184 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 1.84−2.14 (4H, m), 2.92−3.04 (2H, m), 3.29−3.43 (2H, m), 4.01 (1H, d, J = 6.5 Hz), 4.05 (1H, d, J = 6.5 Hz), 4.18 (1H, d, J = 10.2 Hz), 4.33−4.45 (1H, m), 4.49 (1H, d, J = 10.2 Hz), 6.71−6.92 (6H, m), 7.16−7.27 (2H, m), 7.55 (1H, s). MS (DI) *m/z* 484 (M^+). Anal. ($C_{24}H_{25}ClN_4O_5$) C, H, N.

(*R*)-2-{4-[4-(4-Fluorophenoxy)piperidin-1-yl]phenoxymethyl}-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (15). Mp 191−193 °C. ^{1}H NMR (DMSO-d_6) δ 1.59−1.76 (5H, m), 1.92−2.10 (2H, m), 2.80−2.98 (2H, m), 3.24−3.41 (2H, m), 4.10−4.20 (3H, m), 4.37−4.51 (2H, m), 6.78 (2H, d, J = 8.6 Hz), 6.90 (2H, d, J = 8.6 Hz), 6.92−7.12 (4H, m), 8.16 (1H, s). MS (DI) *m/z* 468 (M^+). Anal. ($C_{24}H_{25}FN_4O_5$) C, H, N.

(*R*)-2-Methyl-2-{4-[4-(4-methylphenoxy)piperidin-1-yl]phenoxymethyl}-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (16). Mp 199−201 °C (decomp.). ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 1.86−2.14 (4H, m), 2.29 (3H, s), 2.88−3.04 (2H, m), 3.29−3.45 (2H, m), 4.00 (1H, d, J = 6.3 Hz), 4.04 (1H, d, J = 6.3 Hz), 4.17 (1H, d, J = 10.1 Hz), 4.33−4.43 (1H, m), 4.49 (1H, d, J = 10.1 Hz), 6.71−6.92 (6H, m), 7.08 (2H, d, J = 8.4 Hz), 7.55 (1H, s). MS (DI) *m/z* 464 (M^+). Anal. ($C_{25}H_{28}N_4O_5$) C, H, N.

(*R*)-2-{4-[4-(4-Methoxyphenoxy)piperidin-1-yl]phenoxymethyl}-2-methyl-6-nitro-2,3-dihydroimidazo[2,1-*b*]oxazole (17). Mp 193−195 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 1.82−2.14 (4H, m), 2.86−3.02 (2H, m), 3.31−3.45 (2H, m), 3.77 (3H, s), 4.00 (1H, d, J = 6.2 Hz), 4.04 (1H, d, J = 6.2 Hz), 4.18 (1H, d, J = 10.1 Hz), 4.22−4.35 (1H, m), 4.49 (1H, d, J = 10.1 Hz), 6.71−6.92 (8H, m), 7.55 (1H, s). MS (DI) *m/z* 480 (M^+). Anal. ($C_{25}H_{28}N_4O_6$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-{4-[4-(4-trifluoromethylphenoxy)piperidin-1-yl]phenoxymethyl}-2,3-dihydroimidazo[2,1-*b*]oxazole (18). Mp 179−181 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 1.88−2.20 (4H, m), 2.92−3.10 (2H, m), 3.27−3.43 (2H, m), 4.01 (1H, d, J = 5.8 Hz), 4.05 (1H, d, J = 5.8 Hz), 4.18 (1H, d, J = 10.2 Hz), 4.43−4.57 (2H, m), 6.78 (2H, d, J = 6.8 Hz), 6.90 (2H, d, J = 6.8 Hz), 6.98 (2H, d, J = 8.6 Hz), 7.47−7.60 (3H, m). MS (DI) *m/z* 518 (M^+). Anal. ($C_{25}H_{25}F_3N_4O_5$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-{4-[4-(2-trifluoromethoxyphenoxy)piperidin-1-yl]phenoxymethyl}-2,3-dihydroimidazo[2,1-*b*]oxazole (20). Mp 152−153 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 1.86−2.19 (4H, m), 2.95−3.12 (2H, m), 3.28−3.44 (2H, m), 4.10 (1H, d, J = 10.2 Hz), 4.04 (1H, d, J = 10.2 Hz), 4.18 (1H, d, J = 10.2 Hz), 4.42−4.56 (2H, m), 6.78 (2H, dd, J = 2.3 Hz, 6.9 Hz), 6.83−7.07 (4H, m), 7.14−7.28 (2H, m), 7.55 (1H, s). MS (DI) *m/z* 534 (M^+). Anal. ($C_{25}H_{25}F_3N_4O_6$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-{4-[4-(3-trifluoromethoxyphenoxy)piperidin-1-yl]phenoxymethyl}-2,3-dihydroimidazo[2,1-*b*]oxazole (21). Mp 184−186 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 1.88−2.17 (4H, m), 2.96−3.06 (2H, m), 3.31−3.41 (2H, m), 4.02 (1H, d, J = 10.2 Hz), 4.04 (1H, d, J = 10.2 Hz), 4.18 (1H, d, J = 10.2 Hz), 4.40−4.48 (1H, m), 4.50 (1H, d, J = 10.2 Hz), 6.74−6.94 (7H, m), 7.24−7.31 (1H, m), 7.55 (1H, s). MS (DI) *m/z* 534 (M^+). Anal. ($C_{25}H_{25}F_3N_4O_6$) C, H, N.

(*S*)-2-Methyl-6-nitro-2-phenoxymethyl-2,3-dihydroimidazo-[2,1-*b*]oxazole (3g). This compound was obtained by the same procedure as described for **19** using (*S*)-form epoxide **29** instead of **27**. Mp 153−155 °C. ^{1}H NMR ($CDCl_3$) δ 1.79 (3H, s), 4.04 (1H, d, J = 10.2 Hz), 4.09 (1H, d, J = 10.2 Hz), 4.24 (1H, d, J = 10.1 Hz), 4.50 (1H, d, J = 10.1 Hz), 6.83 (2H, dd, J = 2.0 Hz, 8.6 Hz), 7.01 (1H, t, J = 7.4 Hz), 7.20−7.31 (2H, m), 7.56 (1H, s). MS (DI) *m/z* 275 (M^+). Anal. ($C_{13}H_{13}N_3O_4$) C, H, N.

(*R*)-2-Methyl-6-nitro-2-{4-[(1-oxo-thiomorpholin)-4-yl]phenoxymethyl}-2,3-dihydroimidazo[2,1-*b*]oxazole (12). To a solution of **11** (85 mg, 0.23 mmol) in dichloromethane (5 mL) was added 70% *m*-chloroperbenzoic acid (59 mg, 0.24 mmol), and the resulting mixture was stirred at room temperature for 20 min. Sodium thiosulfate aqueous solution (10%, 15 mL) was added to the reaction mixture, which was extracted with dichloromethane (20 mL). The organic layer was washed with saturated sodium hydrogen carbonate aqueous solution (15 mL) and brine, dried over magnesium sulfate, and filtered. The filtrate was concentrated under reduced pressure. The residue was recrystallized from methanol−isopropylether to afford **12** (59 mg, 67%) as a colorless crystalline powder. Mp 198−200 °C. ^{1}H NMR ($CDCl_3$) δ 1.77 (3H, s), 2.82−2.96 (4H, m), 3.33−3.45 (2H, m), 3.78−3.90 (2H, m), 4.02 (1H, d, J = 5.5 Hz), 4.06 (1H, d, J = 5.5 Hz), 4.20 (1H, d, J = 10.2 Hz), 4.49 (1H, d, J = 10.2 Hz), 6.80 (2H, d, J = 6.8 Hz), 6.91 (2H, d, J = 6.8 Hz), 7.56 (1H, s). MS (DI) *m/z* 392 (M^+). Anal. ($C_{17}H_{20}N_4O_5S$) C, H, N.

***In Vitro* Antituberculosis Activity.** MICs of test agents against both drug-susceptible and drug-resistant strains of *M. tuberculosis* $H_{37}Rv$ were determined essentially according to the previously reported method.[22] Test drugs were each dissolved in dimethyl sulfoxide (DMSO), and the solutions were diluted serially with DMSO in 2-fold dilutions to the desired concentrations. All strains were grown in Middlebrook 7H9 broth. Stock cultures stored frozen at −80 °C were diluted and adjusted to approximately 10^6 CFU/mL. The bacterial suspension containing about 10^6 CFU/mL was spotted onto 7H11 agar plates containing test drugs using a multipoint inoculator (Sakuma Seisakusho). After cultivation at 37 °C for 14 days, MICs were determined as the minimum concentrations of drugs completely inhibiting visible growth of organism.

***In Vivo* Efficacy for 10 Days.** The basic therapeutic efficacy of test agents was determined in mouse models of acute bacterial infection with *M. tuberculosis* Kurono.[10] In brief, the designated compound was suspended in 5% gum arabic. ICR male mice (Japan

SLC) weighing 20−25 g were infected intravenously with 10^4 CFU of mycobacteria through a caudal tail vein and treated once daily at oral doses of 0.5−50 mg/kg for 10 days ($n = 2$) starting on the day after infection. Animals were sacrificed on day 11, approximately 24 h after administration of the final drug dose. Lungs were aseptically removed and ground in a contained tissue homogenizer. The number of viable organisms was determined by dilution plating on 7H11 agar plate and incubating at 37 °C for 14 days prior to counting. Mean log colony forming units (CFU) reduction values were calculated from mycobacterial counts of test groups relative to untreated controls.

***In Vivo* Efficacy for 28 Days.** The designated compound was suspended in 5% gum arabic. ICR male mice (Japan SLC) weighing 20−25 g were infected intravenously with 10^4 CFU of mycobacteria through a caudal tail vein and treated once daily at various oral doses for 28 days ($n = 6$) starting on the day after infection. Animals were sacrificed on day 29, approximately 24 h after administration of the final drug dose. Lungs were aseptically removed and ground in a contained tissue homogenizer. The number of viable organisms was determined by dilution plating on 7H11 agar plate and incubating at 37 °C for 14 days prior to counting. Bacterial counts of test groups were measured and compared with the counts from untreated controls.

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Supporting Information Available: Result of elemental analysis for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Screening for Novel Antituberculosis Agents that are Effective Against Multidrug Resistant Tuberculosis

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Abstract: The challenges in preventing and controlling tuberculosis are further complicated by the deadly rise of multi-drug-resistant tuberculosis (MDR-TB). Recognizing the seriousness of the situation, we initiated a program to screen new agents that would satisfy these unmet needs and have a favorable safety profile. Mycobacteria are well known for their lipid-rich properties. In *Mycobacterium tuberculosis*, mycolic acid in particular has been established the wall component related to the pathogenesis in the host. There are approximately 250 identified genes related to biosynthesis of the lipid turnover that contain InhA, the main target of isoniazid. Thus, the logical approach for developing a chemotherapy agent against tubercle bacilli included screening compounds that could inhibit the biosyntheses of mycolic acid and that had a novel chemical structure to ensure improved efficacy against MDR-TB. Some of the screening systems established for those purposes and some of the candidates are outlined.

Keywords: Tuberculosis, mycolic acid inhibitor, nitrodehydroimidazooxazole, mycolic acid, BRM test.

INTRODUCTION

Among the various infectious diseases seen worldwide, tuberculosis (TB) remains the disease that inflicts the highest death toll [1]. In 1993, the World Health Organization declared TB to be a global emergency. Reportedly, 32% of the world's population are currently infected with *Mycobacterium tuberculosis*, and each year 8 million people develop active TB and 2 million die as a result [2]. The annual rate of increase in TB incidence is 3% globally, but is as high as 7% in Eastern Europe and higher than 10% in some African countries [3].

The situation is further fuelled by the deadly rise of multidrug-resistant TB (MDR-TB). Epidemics of MDR-TB can spread quickly from city to city, from country to country, and even from continent to continent. A survey of 72 countries suggested that the problem of MDR-TB is more widespread than previously thought and is most likely worsening [4], and some experts estimate that between 185,000 and 415,000 new cases of MDR-TB develop each year [5].

The current TB drug regimen has several disadvantages, and there a number of desirable qualities that a new anti-TB drug should have. The first is shortened treatment duration. Currently, patients require between 6 and 9 months of treatment. This long treatment period leads to lack of compliance, which in turn can be responsible for relapse and the emergence of MDR-TB strains. With MDR-TB posing a major threat, a new TB drug would have to also address this issue. It would also be desirable for the new drugs to have fewer side effects than existing chemotherapeutics, thus providing better overall safety. We have carried out our TB research program with these expectations in mind. When patients present at the clinic, they typically start out with a regimen of 4 drugs – isoniazid, rifampicin, pyrazinamide, and either streptomycin or ethambutol – for the first 2 months in the initial phase, followed by isoniazid and rifampicin for 4 months in the continuation phase. During treatment, the tubercle bacilli are still multiplying, although at a slower rate. During this process, mutant bacilli resistant to one or more of the drugs can emerge and cause the patient to relapse with MDR-TB.

The action mechanisms of the conventional TB drugs illustrated in Fig. (1) provide valuable information as to their resistance mechanisms [6,7,8,9,10]. Isoniazid is activated by KatG to form a complex with NAD, which then inhibits InhA [6]. Thus, mutation of either the Kat G gene or the InhA gene produces isoniazid resistance. Since the target of rifampicin is the RNA polymerase beta subunit, mutation in the *rpoB* gene gives rise to rifampicin resistance [7]. Bacteria resistant to both isoniazid and rifampicin, formally designated as MDR-TB, have been increasing and spreading worldwide.

STRATEGY

Recognizing the seriousness of the situation, we initiated a program to screen for new antituberculosis agents that would satisfy these unmet needs. To establish a strategy for our approach, we looked at the process from infection to disease for tuberculosis as illustrated in Fig. (2). Infection typically occurs by inhaling airborne *Mycobacterium tuberculosis*. Within the first two years, 5% of newly infected individuals develop the disease. The tubercle bacilli survive as "persisters" in the remaining individuals, and of those about 5% contract active TB at some point later in life, but 90% never develop the disease. These phenomena suggest 2 broad areas of approach: the more common approach is to target the pathogen, which is done through administering antimicrobial agents, and the other is to target the host by stimulating the self-defense system through

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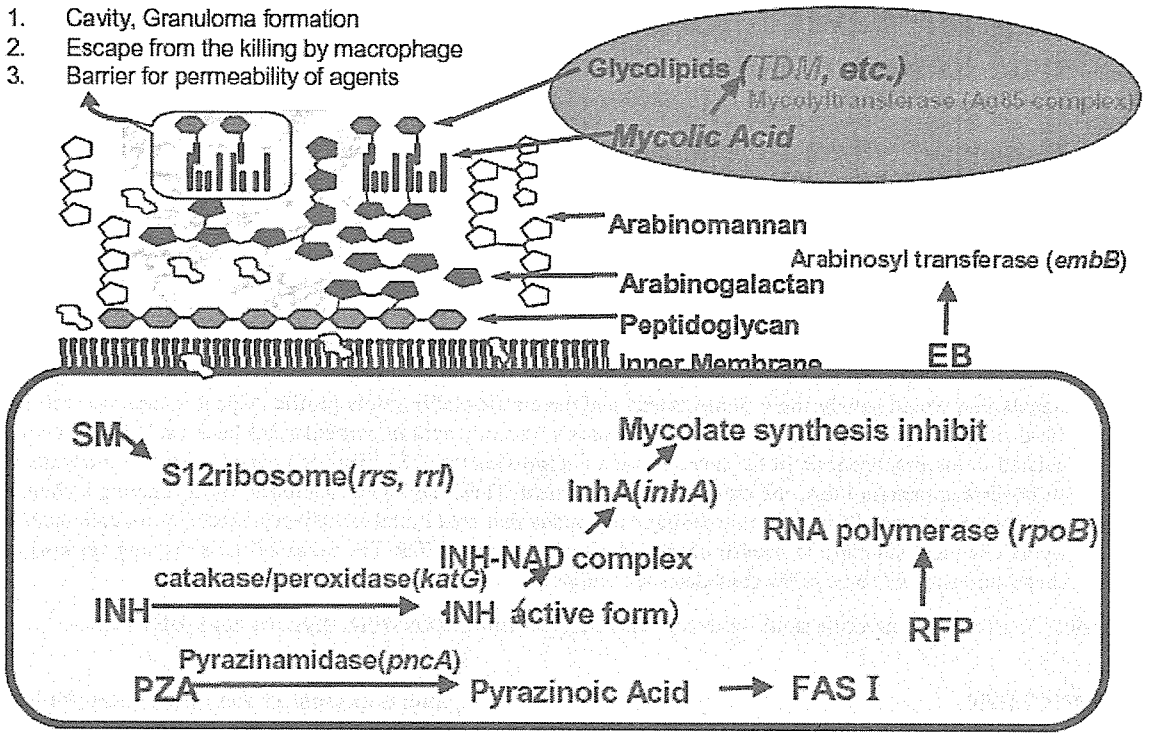


Fig. (1). Mechanism of Action of Conventional TB Drugs.

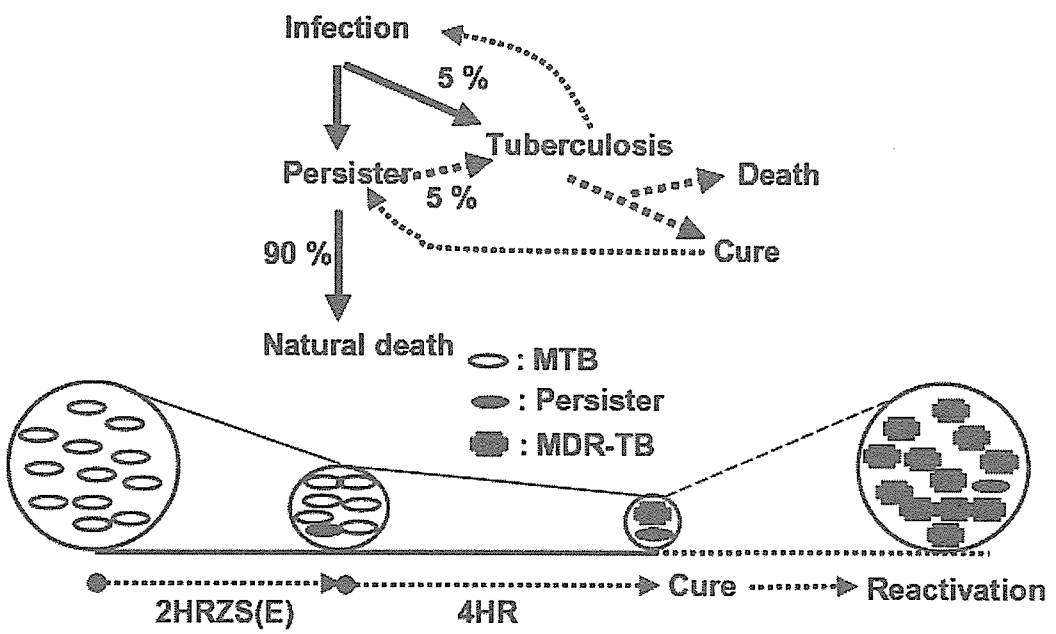


Fig. (2). The Tuberculosis Disease Process and the Emergence of MDR-TB.

vaccines, gene therapy, immunotherapy, and cytokine therapy [11, 12, 13, 14]. An overall approach for TB therapy should include a delicate balance between these two approaches.

For improved efficacy against MDR-TB, we screened for compounds with a new structure and mechanism of action. Mycobacteria are well known to be wax-rich bacteria, and a main component of the wax is mycolic acid, which is present only in mycobacteria and not in gram-positive or gram-negative bacteria or in mammalian cells. Genomic analysis has verified the lipid richness of tubercle bacilli, showing that there are almost 250 distinct enzymes involved in the lipid metabolism of tubercle bacilli [15]. Glycolipids such as trehalose dimycolate are well known as a virulence factors related to cavity and granuloma formation, survival in macrophages, and a barrier to the permeability of drugs [16, 17, 18]. We thus regarded the mycolic acid cell-wall component as an ideal target for a new agent, not only for

exerting antituberculosis activity but also for decreasing the virulence of the bacteria. In view of the important role of mycolic acids in mycobacteria, we screened for inhibitors of mycolic acid synthesis that had potent antibacterial activity.

INHIBITORS OF MYCOLIC ACID BIOSYNTHESIS

Using random screening, we found several compounds that inhibited mycolic acid biosynthesis. Some of the structures of the hit compounds are shown in Fig. (**3**). Compound A in particular possessed a unique profile. Many derivatives of this compound were then synthesized, and one candidate, OPC-37306, was brought forward as a candidate for further evaluation.

A profile summary of OPC-37306 is shown in Fig. (**4**). The compound is a newly synthesized dihydrophenazine derivative that showed dose-dependent inhibitory activity against mycolic acid biosynthesis (Fig. (**4b**)) and exhibited potent *in vitro* antituberculosis activity, with minimal inhibitor concentrations (MICs) in the range of 0.1 to 0.2 μg/mL (Fig. (**4c**)). Interestingly, this compound did not kill gram-positive or gram-negative bacteria (data not shown), likely because of its inhibition of mycolic acid biosynthesis, which is specific to mycobacteria. We then examined whether this compound could show potency in an experimental tuberculosis model in mice. The results indicated OPC-37306 to have dose-dependent therapeutic efficacy that was more potent than that of rifampicin.

Other structures of interest that we focused on were urea-type derivatives and dihydroimidazooxazole derivatives. These compounds also showed TB-specific activity that included drug resistant bacteria through an inhibition of mycolic acid biosynthesis. The *in vitro* antituberculosis activity of the urea-type derivatives was potent, with MIC values of around 0.1μg/ml, and these derivatives also did not show any activity against gram-positive and gram-negative bacteria. The urea-type derivatives, however, did not show any therapeutic efficacy in an experimental tuberculosis mouse model. The reason for the lack of efficacy has not been clarified, but it could be due to reasons such as poor absorption and high protein binding.

OPC-J is one of the dihydroimidazooxazole derivatives that inhibited mycolic acid biosynthesis. This compound also demonstrated mycobacteria-specific antituberculosis activity and did not show any activity against gram-positive or gram-negative bacteria. Nitroimidazole-type compounds, which are based on the structure of nitro-heterocyclic compounds including various 5- and 2-nitroimidazoles and 5-nitrofurans, are known to be effective against a number protozoan and bacterial infections in humans and animals [19]. These compounds, however, are also known to commonly possess mutagenicity, which poses a major hurdle to their development. CGI is a nitroimidazole that showed potent antituberculosis activity but was not developed because of its mutagenicity [20, 21].

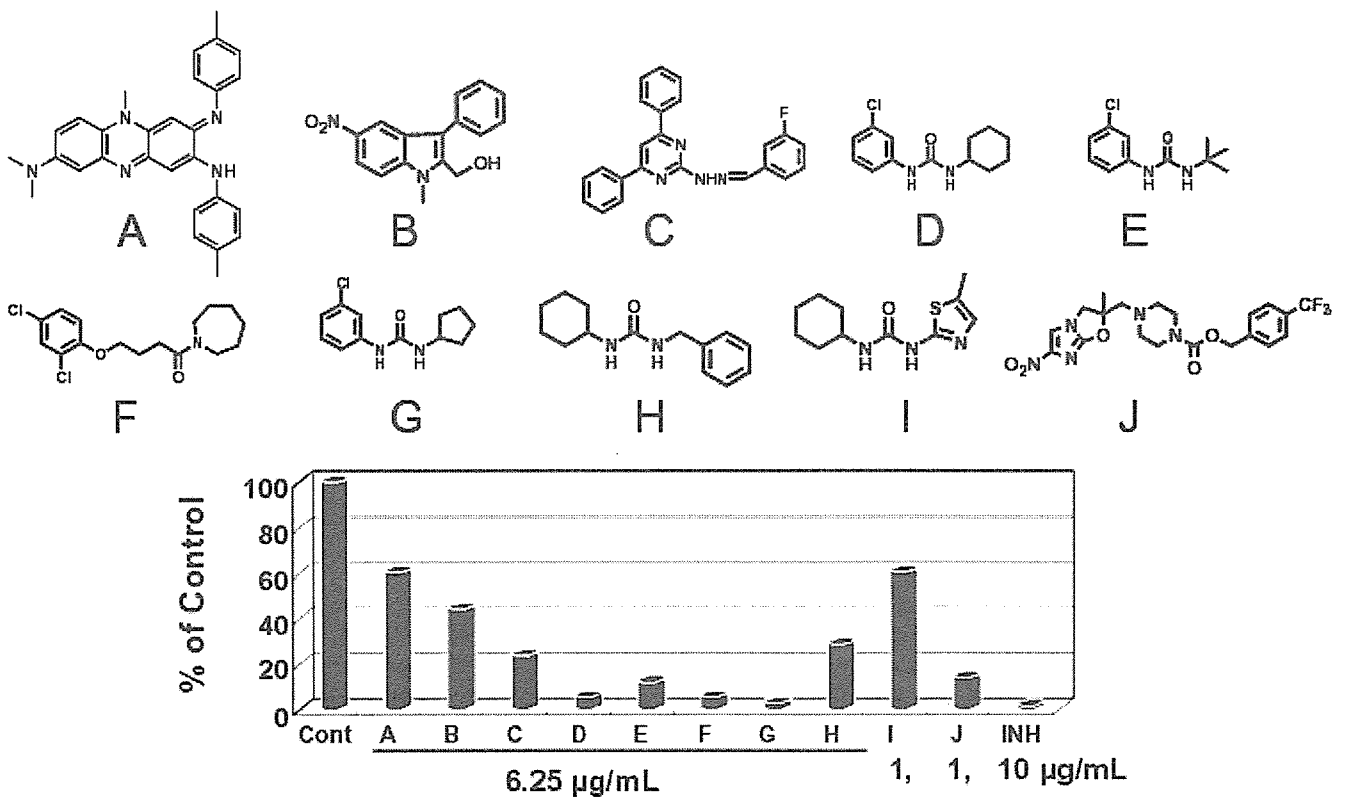
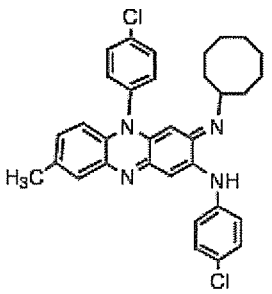
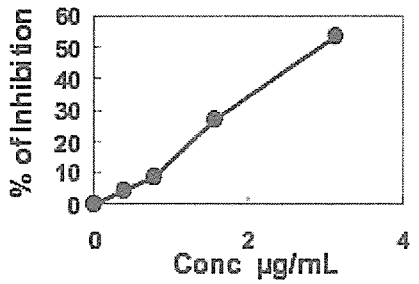


Fig. (3). Inhibitors of Mycolic Acid Biosynthesis: The compound A, B, C, D, E, F, G, H, I, and J showing the inhibitory activity against mycolic acid biosynthesis in Mycobacteria was randomly selected and compared with that of INH, a well-known inhibitor of mycolic acid synthesis. ^{14}C-labeled acetic acid was incorporated to mycolic acid by incubation with *M. bovis* BCG cell cultures in the presence of 6.25 μg/ml of A, B, C, D, E, F, G, or H, or 1μg/ml of I, J, or INH. ^{14}C-labeled fatty acid and mycolic acid s were detected using thin-layer chromatography, and analyzed by BAS-2500 (Fujifilm). The radioactivity of each fatty acid and mycolic acid was calculated using PhotoStimulated Luminescence, expressed as the percentage of incorporation in untreated controls.

a



b



c

Strains	MIC μg/mL
M.tuberculosis H37Rv	0.2
M.tuberculosis H37Rv RFPr	0.2
M.tuberculosis H37Rv INHr	0.2
M.tuberculosis H37Rv EBr	0.1
M.tuberculosis H37Rv SMr	0.1
M.tuberculosis Kurono	0.1
M.bovis BCG	0.2

d

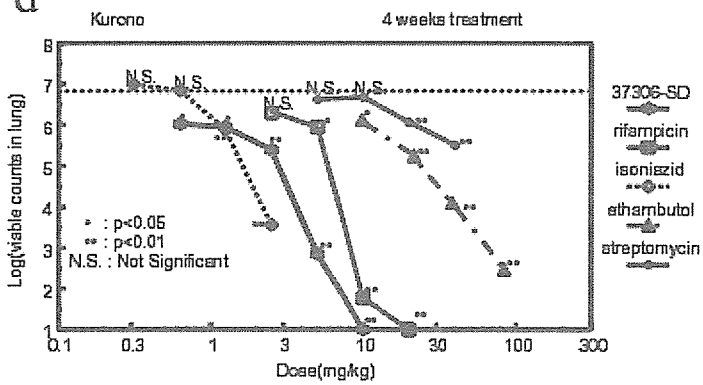


Fig. (4). Antituberculosis Profile of OPC-37306

(a) Structure of OPC-37306. (b) Inhibitory activity against mycolic acid biosynthesis; ^{14}C-labeled acetic acid was incorporated to mycolic acid by incubation with Mycobacteria cell cultures in the presence of 0.39, 0.78, 1.56, 3.13 μg/ml of OPC-37306. ^{14}C-labeled fatty acid and mycolic acids were detected using thin-layer chromatography, and analyzed by BAS-2500 (Fujifilm). The radioactivity of mycolic acids were calculated using PhotoStimulated Luminescence, expressed as the percentage of incorporation in untreated controls. Percent of inhibitory activity was plotted in the figure. (c) Susceptibility test; The *in vitro* antimycobacterial activity of the OPC-37306 against *M. tuberculosis* H37Rv, H37Rv RFPr (resistance to RFP), H37Rv INHr (resistance to INH), H37Rv EBr (resistance to EB), H37Rv SMr (resistance to SM), Kurono, and *M.bovis* BCG were examined by an agar dilution method using 7H11 agar plates. The MIC was determined as the lowest concentration that inhibited visible growth of the organism on the agar medium after incubation. (d) Therapeutic efficacy; ICR mice (n=5) were inoculated intravenously with 0.2 ml of suspension containing *M. tuberculosis* Kurono at 10^4 CFU/ml. The designated compound (OPC-37306: 0.625-10 mg/kg, RFP: 2.5-20 mg/kg, INH: 0.313-1.56 mg/kg, EB: 10-80 mg/kg, SM: 5-40 mg/kg mg/kg) suspended in 5% gum arabic or saline for SM was then administered orally once daily for 4 weeks. At the end of the treatment period, the mice were euthanized (exsanguination through the abdominal inferior vena cava) under ether anesthesia, and the lung was aseptically excised. A lung homogenate for each mouse was prepared by pestling the lung evenly with a glass homogenizer after adding sterile distilled water to the excised lungs, and the homogenate was then diluted further with distilled water. A smear plate for each lung homogenate was then prepared by spreading 0.1 ml of each diluted solution on a 7H11 agar plate using a spreader. All plates after spreading the homogenate solution were incubated at 37℃ and counted for formed colonies after 14 days. Statistical analysis was conducted using SAS software (SAS Institute Japan, R. 8.1) on the number of viable bacteria in the lung of mice surviving until necropsy on the 29th day after inoculation. The significance level of the test was set at 5%. A test for dose dependency was performed using linear regression analysis based on log-transformed values of the viable bacterial counts in the lung. When dose dependency was confirmed, the Williams test (lower-tailed) was subsequently performed, and when dose dependency was not confirmed, the Dunnett's test (two-tailed) was subsequently performed against each of the control groups. The care and handling of the animals was in accordance with "Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co., Ltd."

Investigation into the mutagenicity of these compounds has revealed that the mutations occur as a result of damage to the DNA by O_2 and NO radicals that are formed during metabolism of the nitro residue. One seemingly logical means to avoid the mutations would be to prevent the nitrogen from being metabolized, but there is no theoretical way to modify the structure in such a manner.

To overcome the hurdles associated with mutagenicity, we looked at the possibility of avoiding the problem by testing all of the nitroimidazole derivatives in our library using the Ames test. The strains used in the Ames test were *Salmonella typhimurium* TA98 (frame shift), *Salmonella typhimurium* TA100 (GC base pair), *Salmonella typhimurium* TA1535(GC base pair), *Salmonella typhimurium* TA1537(GC base pair), and *Escherichia coli* wp2 (frame shift). Using this method we observed that the number of revertant colonies formed on the plates varied with the compound structures. Some of these data are summarized in Fig. (**5**). These findings appear to indicate that the mutagenic potency of the nitrodihydroimidazooxazole-based compounds is dependent on the functional groups attached to the core structure. Most of the nitroimidazole derivatives we tested showed mutagenicity,

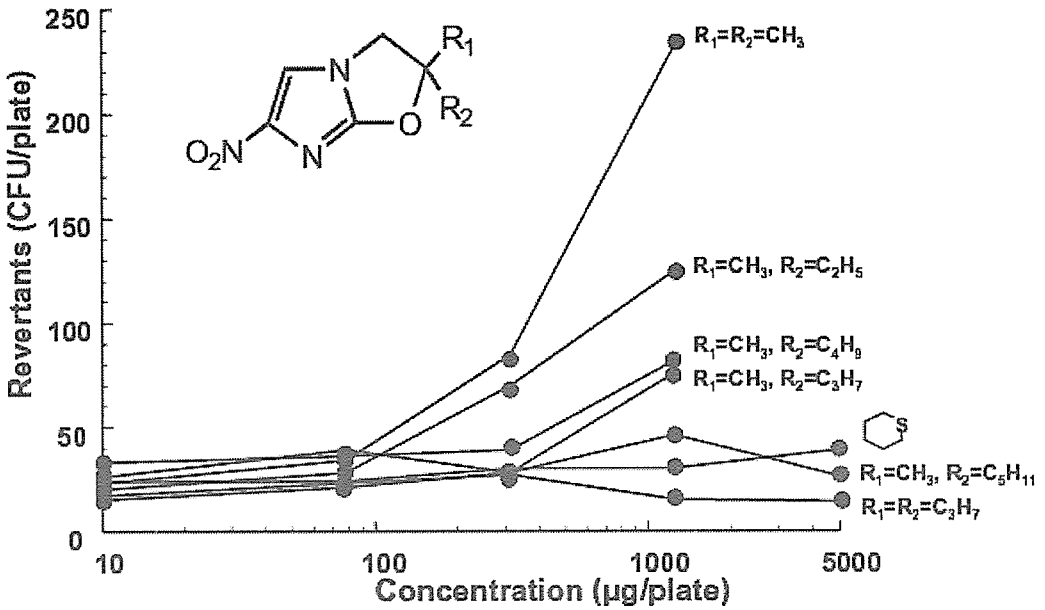
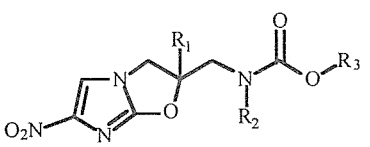
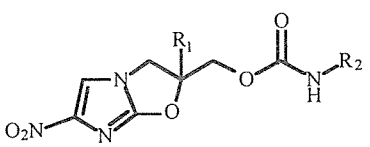
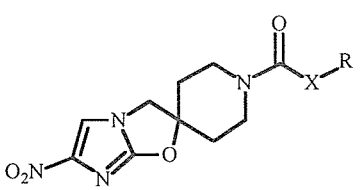
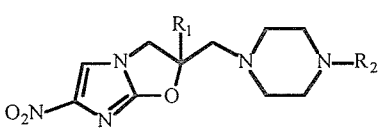
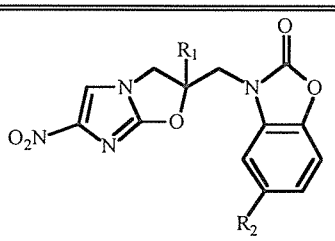
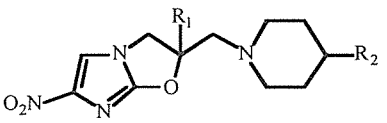
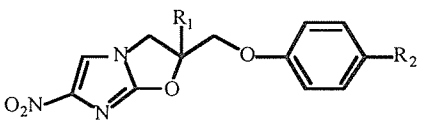


Fig. (5). Ames Test for the Dihydroimidazooxazole Derivatives: The BRM test was performed in accordance with OECD Guideline 471 using *Salmonella tyiphimurium* TA98. Each bacterial strain was precultured at 37°C for 18 h using a nutrient broth (Nissui). After adjustment to 2.4 at OD660 nm, each bacterial suspension was added to a test tube containing the designated compound. After a 20-minute incubation at 37°C, top agar was added to each test tube and the contents were poured into minimum essential medium (Oriental Yeast). The number of revertants was counted 48 h after incubation at 37°C.

Table 1. Relationship between Ames Test and MIC Values For Selected Dihyrdoimidazooxazoles

	Ames-	MIC mg/mL
	67%	0.2 – 25
	100%	0.39 – 1.56
	94%	0.39 -> 100
	83%	0.012 – 3.13

(Table 1) Contd....

	Ames-	MIC mg/mL
	100%	0.39
	98%	<0.006 – 1.56
	58%	0.012 – 1.56

Ames test: BRM test was performed in accordance with OECD Guideline 471 using *Salmonella tyiphimurium* TA98, TA100, TA1535, TA1537, and TA102 or *Escherichia coli* WP2*uvrA*. The data are expressed as Ames-negative percentage among the tested compounds.

Susceptibility test: The in vitro antimycobacterial activity of the compounds against *Mycobacterium tuberculosis* H37Rv were examined and evaluated by an agar dilution method using 7H11 agar plates. The MIC was determined as the lowest concentration that inhibited visible growth of the organism on the agar medium after incubation. The data are shown as the range of the MIC among each structural type.

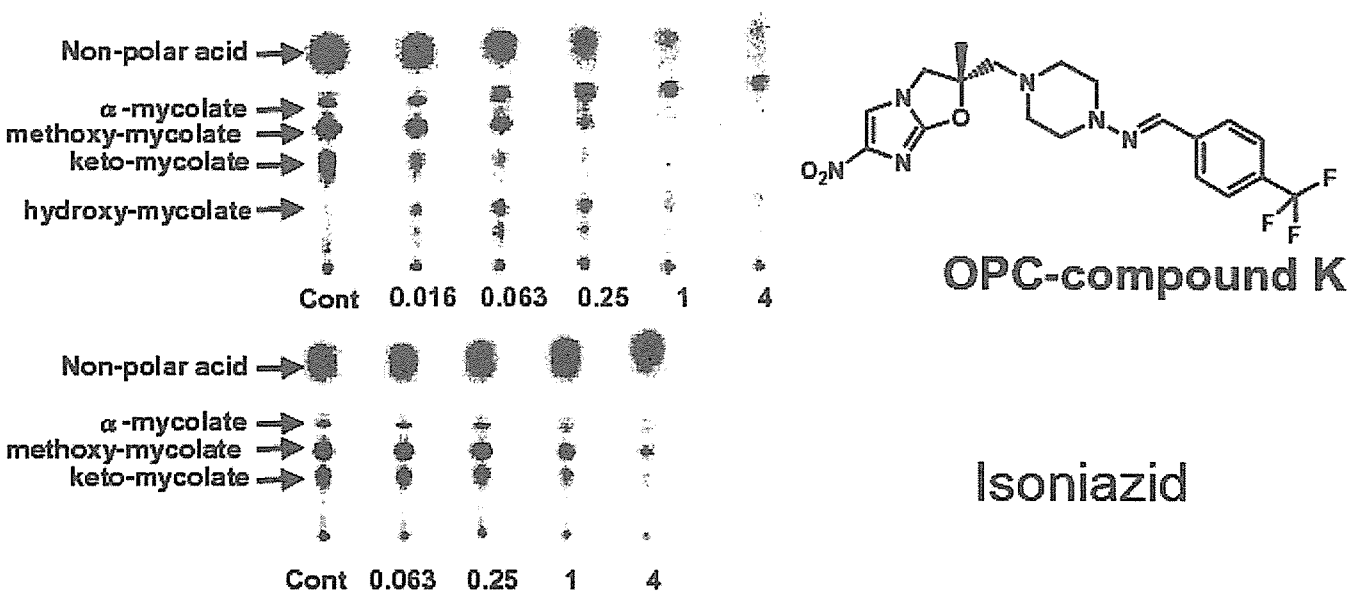


Fig. (6). Inhibitory Activity of Compound K and Isoniazid on the Biosynthesis of Mycolic Acids in the BCG Strain: The inhibitory activity of the compound K against mycolic acid synthesis in *M. bovis* BCG was determined and compared with that of INH, a well-known inhibitor of mycolic acid synthesis. ^{14}C-labeled acetic acid was incorporated to mycolic acid by incubation with *M. bovis* BCG cell cultures in the presence of compound K or INH as a reference. ^{14}C-labeled fatty acid and mycolic acid subclasses were detected using thin-layer chromatography, and analyzed by BAS-2500 (Fujifilm).

particularly those with dimethyl residues. However, 22% of the tested compounds did not show mutagenicity, providing a hint to help us overcome the hurdles associated with mutagenicity.

We then introduced various types of residues to the dihydroimidazooxazole structures and evaluated their mutagenicity and *in vitro* antituberculosis activities. Carbamate-type residues overall were 67% Ames negative, but those

residues that exchanged nitrogen for oxygen were 100% Ames negative. Introduction of spiral-, piperazine-, heterocylic-, phenoxy-, and piperidine-type residues produced respective Ames negativity results of 94%, 83%, 100%, 98%, and 58%. In terms of antituberculosis activity, piperazine-, piperidine-, and phenoxy-type residues all showed high potency, with phenoxy-type residues producing the best overall results. A summary of the results are shown in Table (**1**).

Our optimized imidazooxazole compound OPC-K inhibited the biosynthesis of mycolic acid dose dependently, as also shown for isoniazid. However, while isoniazid inhibited the synthesis of alpha, methoxy, and keto mycolic acids, OPC-K only affected the synthesis of methoxy and keto mycolic acids (Fig. **6**). OPC-K showed potent *in vitro* antituberculosis activity against standard TB strains,, with MICs ranging from 0.025 to 0.39μg/ml (Table **2**). The *in vivo* therapeutic efficacy of OPC-K was also evaluated, and the results showed OPC-K to be more potent than rifampicin (Fig. (**7**)).

Table 2. Minimum Inhibitory Concentrations of Compound K Against *M.tuberculosis*

Strains	MIC mg/mL
M. tuberculosis H37Rv	0.39
M. tuberculosis H37Rv RFPr	0.025
M. tuberculosis H37Rv INHr	0.2
M. tuberculosis H37Rv EBr	0.2
M. tuberculosis H37Rv SMr	0.39
M. tuberculosis Kurono	0.2
M. bovis BCG	0.05

Susceptibility test: The *in vitro* antimycobacterial activity of the compound K against *M. tuberculosis* H37Rv, H37Rv RFPr (resistance to RFP), H37Rv INHr (resistance to INH), H37Rv EBr (resistance to EB), H37Rv SMr (resistance to SM), Kurono, and *M.bovis* BCG were examined by an agar dilution method using 7H11 agar plates. The MIC was determined as the lowest concentration that inhibited visible growth of the organism on the agar medium after incubation.

The relationship between the inhibition of mycolic acid subclasses and the MIC values was evaluated to confirm whether the target of these compound series is the metabolism of biosynthesis of mycolic acid. The results indicated that antituberculosis activities were well correlated to the inhibitory activity of methoxy and keto mycolic acid biosynthesis but not for alpha-mycolic acid or fatty acid (Fig. (**8**)). We thus concluded that a mechanism involved in the antituberculosis activity of the compound deals with the inhibition of mycolic acid.

CONCLUSIONS

We successfully synthesized nitro-imidazooxazole derivatives that were free from mutagenicity by introducing unique residues and found no correlation between mutageneticity and antituberculosis potency (Fig. (**9**)). Based on these results, we were convinced that dihydroimidazooxazole derivatives could be a candidate for the development of a new-class of TB agents effective against MDR-TB.

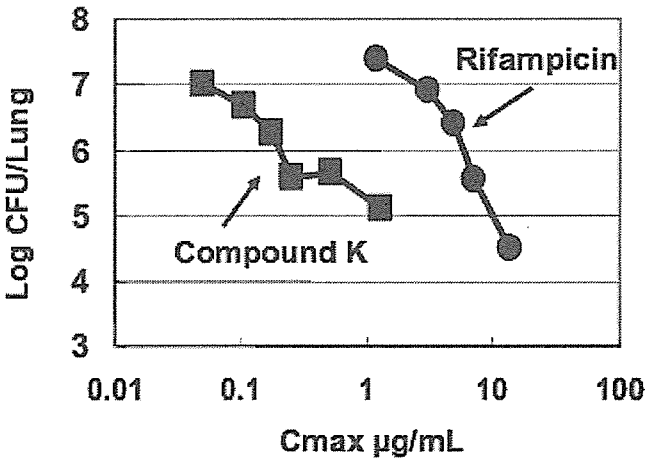


Fig. (7). Therapeutic Efficacies of Compound K and Rifampicin in an Experimental Tuberculosis Mouse Model: Therapeutic efficacy; ICR mice (n=5) were inoculated intravenously with 0.2 ml of suspension containing *M. tuberculosis* Kurono at 10^4 CFU/ml. The designated compound (Compound K: 0.039, 0.078, 0.156, 0.313, 0.625, and 1.25 mg/kg, RFP: 1.25, 2.5, 5, 10, and 20 mg/kg) suspended in 5% gum arabic was then administered orally once daily for 4 weeks. At the end of the treatment period, the mice were euthanized (exsanguination through the abdominal inferior vena cava) under ether anesthesia, and the lung was aseptically excised. A lung homogenate for each mouse was prepared by pestling the lung evenly with a glass homogenizer after adding sterile distilled water to the excised lungs, and the homogenate was then diluted further with distilled water. A smear plate for each lung homogenate was then prepared by spreading 0.1 ml of each diluted solution on a 7H11 agar plate using a spreader. All plates after spreading the homogenate solution were incubated at 37℃ and counted for formed colonies after 14 days. The care and handling of the animals was in accordance with "Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co., Ltd."

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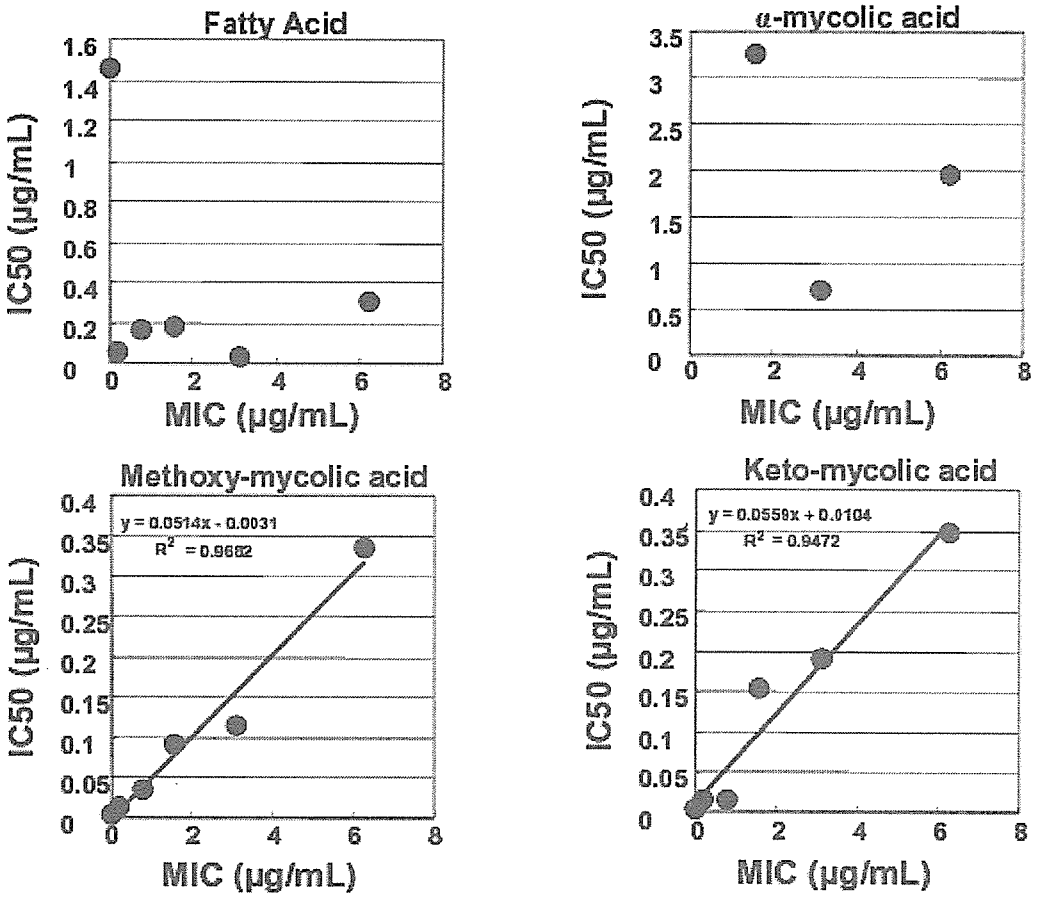

Fig. (8). Relationship Between Mycolic Acid Biosynthesis and MIC Values
Compounds, which show a different MIC value, were randomly selected, and IC_{50} value (concentration required to inhibit by 50%) of each compound was calculated by linear regression analysis based on an inhibitory activity of each mycolic acid subclass and fatty acid biosynthesis. And these values were plotted on the figures to analysis a relationship between the inhibitory activity against mycolic acid biosynthesis and the MIC value.

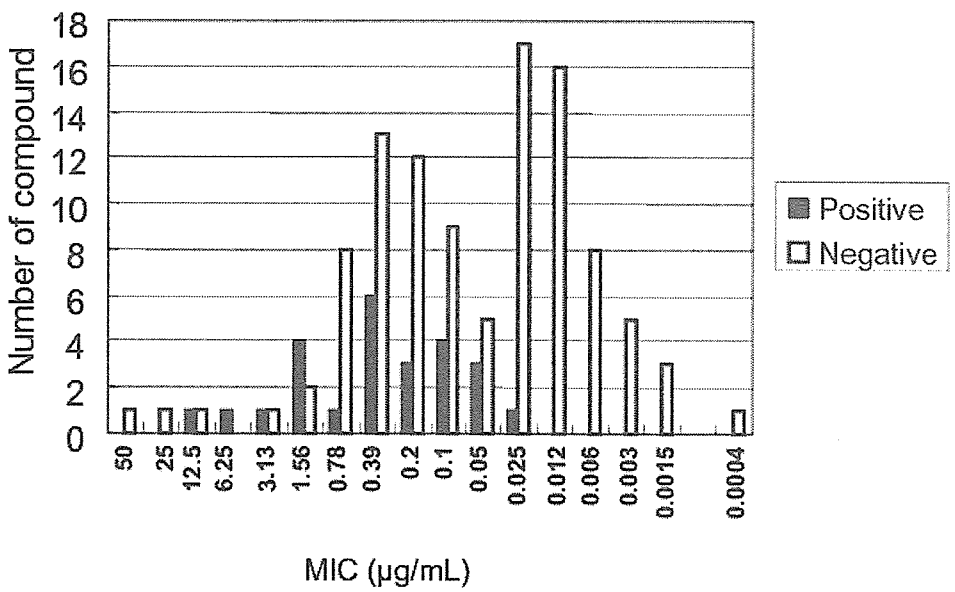

Fig. (9). Relationship Between Mutagenicity and Antimycobial ActivityInsert here footnotes for Figure, showing experimental conditions BRM test was performed in accordance with OECD Guideline 471 using *Salmonella tyiphimurium* TA98, TA100, TA1535, TA1537, and TA102 or Escherichia coli WP2uvrA in the absence and presence of S9-mix.The number of mutagenic or non-mutagenic compounds which showed respective MIC values was plotted.

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7．新たな抗結核薬開発の必要性と世界の現状

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