ンピシン保存溶液)。後はバクテックMGIT 960結核菌薬剤感受性試験用ミジットシリーズの説明書に記載されたプロトコールに従い，菌液500 μlと希釈されたリファンピシン保存溶液100 μlを通常のRIFと表示されたミジットチューブに無菌的に添加した。バクテックMGIT 960にて37℃で培養し，陽性を示した最高濃度の菌液を用いて再度希釈系列にて培養を継続した。最終的にプロスミックNTMのRFP感受性検査でMIC値が32 $\mu g/ml$以上の値を示すまで継代培養を続け，耐性コントロールとした。

PCRによる*rpoB*遺伝子増幅

小川培地からエーゼで2～3 mm径コロニー2個分を目安として採取し，1.5 mlマイクロチューブに分注したインスタジーンDNA精製マトリックス(BIO-RAD) 200 μlに懸濁した。56℃，15～30分処理後10秒間vortexし，正確に100℃，8分間処理した後直ちに放冷した。10秒間vortexし，12000 rpm，3分遠心した上清をpolymerase chain reaction (PCR)に用いた。*rpoB*遺伝子増幅のために，次のプライマーを使用した；MK1：5'-GCG GAT GAC CAC CCA GGA CG-3' とMK2：5'-GCG CGG TCC TC[C/T] TCG TCG GC-3'。PCR条件は95℃ 3分の熱変性の後，94℃ 1分，60℃ 1分，72℃ 1分を30サイクル行った。最後に72℃ 7分間伸張した。得られたPCR産物は，1.5%アガロースゲル電気泳動で確認した。

PCR産物のDNAシークエンス

*rpoB*遺伝子の塩基配列は，290 bpのPCR産物を用いてBigDye Terminator v1.1 Cycle sequencing Kit (ABI)にて決定した。

フィノスLiPA Rif TB

フィノスLiPA Rif TB (ニプロ)は抗酸菌から抽出，増幅されたビオチン化DNAを用いて，結核菌群の*rpoB*遺伝子内の変異を検出するLine Probe Assayである[6]。10種類のプローブを固相化したストリップにNaOH変性した検体を添加して，ハイブリダイズする。洗浄後，ビオチン－アビジン結合を行い，基質(NBT/BCIP)を用いた発色反応から，検体が結合したプローブ部位が発色する。発色したプローブの位置から，結核菌群の検出ならびに*rpoB*遺伝子内の変異の有無の判定を行う。今回*M. kansasii*に対しても結核菌同様，同キットによる*rpoB*変異の検出が可能かどうか検討した。

結　果

薬剤感受性試験のRFP耐性判定基準値は，結核菌に準拠したウエルパック法では40 $\mu g/ml$だが，プロスミックNTM法では，National Committee for Clinical Laboratory Standards (NCCLS：現Clinical Laboratory Standards Institute [CLSI])の判定基準から1 $\mu g/ml$とした[7][8]。ウエルパック法，プロスミックNTM法ともにRFP耐性と判定された*M. kansasii*は314株中3株であった。菌株Aはウエルパック法，プロスミックNTM法共に耐性と判定され，MIC値は2 $\mu g/ml$だった。菌株B，Cは，両薬剤感受性試験で耐性と判定され，MIC値はそれぞれ16 $\mu g/ml$と32 $\mu g/ml$であった。菌株KCHK1001SはRFP感受性(MIC値0.06 $\mu g/ml$)であった(Table)。また今回ウエルパック法とプロスミックNTM法の間でRFP感受性結果の相違は認められなかった。

シークエンス解析の結果，菌株KCHK1001Sの*rpoB*遺伝子のhot spot領域(69 bp)の塩基配列は結核菌の塩基配列と87%の相同性が見られ(GenBank #L27989)，すでに報告されている*rpoB*遺伝子の塩基配列と同一であった(GenBank #AF060301)。

薬剤感受性試験でRFP感受性と判定された*M. kansasii* 45株についてシークエンス解析を実施した結果，*rpoB*遺伝子変異を認めなかった。しかしRFP耐性*M. kansasii*の*rpoB*遺伝子の塩基配列は薬剤感受性試験でRFP耐性となった*M. kansasii* 3株および，菌株KCHK 1001Rすべてに変異を認めた。菌株Aはコドン516においてアスパラギン酸からアラニンへの変異を認めた(GAC→GCC)。菌株B，Cはコドン513においてグルタミンからグルタミン酸への変異を認めた(CAG→GAG)。菌株KCHK1001Rはコドン526においてヒスチジンからアルギニンへの変異を認めた(CAC→CGC) (Fig. 1)。

フィノスLiPA Rif TBの結果，RFP感受性ならびにRFP耐性*M. kansasii*，KCHK1001S，KCHK1001Rすべて

Table Results of RFP susceptibility testing and sequences

Strains	Wellpack*	BrothMIC NTM (MIC)	Sequence
A	RFP resistant	R (2 $\mu g/ml$)	codon 516 (GAC → GCC)
B	RFP resistant	R (16 $\mu g/ml$)	codon 513 (CAG → GAG)
C	RFP resistant	R (32 $\mu g/ml$)	codon 513 (CAG → GAG)
KCHK1001R	RFP resistant	R (32 $\mu g/ml$)	codon 526 (CAC → CGC)
KCHK1001S	RFP susceptible	S (0.06 $\mu g/ml$)	

*Ogawa medium based drug susceptibility test
RFP: rifampicin R: rifampicin resistance S: rifampicin susceptible

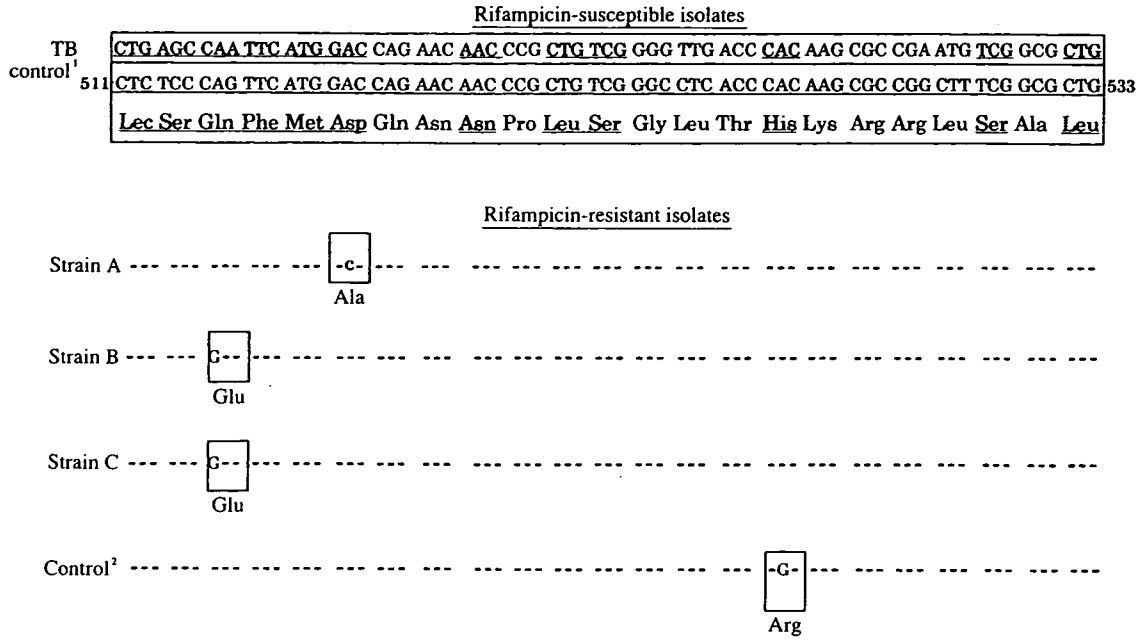

Fig. 1 *rpoB* gene sequences of one rifampicin-susceptible and four rifampicin-resistant strains of *Mycobacterium kansasii*, with the *Mycobacterium tuberculosis* (*M. tuberculosis*) sequence shown for comparison.
1: KCHK1001S (rifampicin-susceptible control)
2: KCHK1001R (rifampicin-resistant control)
Underline codons in *M. tuberculosis*: common codons involved in rifampicin-resistance strains

においてTBプローブ，野生型（S）ならびに変異型（R）プローブに発色を示さなかった（Fig. 2）。

考　察

結核菌のRFP耐性化には*rpoB*遺伝子の突然変異が強くかかわっており，RFP耐性結核菌の約95%が，βサブユニットをコードしている*rpoB*遺伝子のhot spot領域に変異を認めている[9]。今回検討したRFP耐性*M. kansasii*についても，結核菌と同じhot spot領域に*rpoB*遺伝子変異が確認され，RFP感受性*M. kansasii*は*rpoB*遺伝子変異が認められなかった。Kleinらは，RFP耐性*M. kansasii*の*rpoB*遺伝子変異は，結核菌のRFP耐性に強く関与が証明されている*rpoB*遺伝子変異と同じ領域に存在し，*M. kansasii*についても*rpoB*変異とRFP耐性とに強い関連性があると報告している[5]。今回の*rpoB*変異のシークエンス解析で，菌株B，Cはコドン513の変異があり，Kleinらと同じ遺伝子変異をもったタイプであったが，菌株Aはコドン516の変異をもち，Kleinらとは違う変異部位であった。また，Kleinらは臨床菌株3例，環境分離菌株1例にコドン531の変異が見られたと報告しているが，今回われわれの検証では，コドン531の変異は認められなかった。コドン531は結核菌で頻繁に変異しやすい部位であることから[10]，今後データの蓄積によりコドン531に変異をもった菌株や，異なる変異部位を

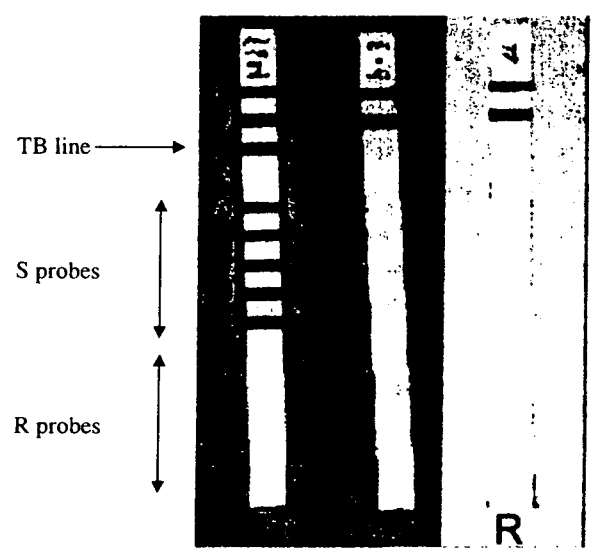

Fig. 2 The patterns of rifampicin-susceptible and resistant strains of *M. kansasii* by Line Probe Assay.
TB: *M. tuberculosis* (H37Rv) indicated the reaction of the TB line and the five S probes
S: KCHK1001S (rifampicin-susceptible strain of *M. kansasii*)
R: KCHK1001R (rifampicin-resistant strain of *M. kansasii*)

もった違うタイプの *M. kansasii* の存在も認められるであろう。またわれわれが作成した菌株 KCHK1001R の変異はコドン526であった。Klein らが作成した誘導 RFP 耐性 *M. kansasii* は今回われわれが MGIT 960結核菌薬剤感受性検査用ミジットシリーズのリファンピシン感受性検査用チューブを用いた方法とは違い，ミドルブルック7H11培地を用いた手法で作成されているが，同じコドン526の部位に変異をもっていた[5]。以上のことから *in vitro* で RFP 耐性を獲得した *M. kansasii* は，コドン526の遺伝子部位に変異を起こしやすい可能性が考えられる。同様に，菌株 KCHK1001S ならびに RFP 感受性 *M. kansasii* 45株はすべてシークエンス解析において hot spot 領域に *rpoB* 遺伝子変異を認めなかったことから，RFP 感受性試験結果と *rpoB* 遺伝子変異の強い関連性が示唆された。

また，結核菌群と同じ69 bp の hot spot 領域に変異をもつ RFP 耐性 *M. kansasii* が結核菌群と同じ遺伝子変異をもつならば，結核菌群に特異的なプローブと反応する可能性がある。そこでわれわれは結核菌群の *rpoB* 遺伝子の hot spot 領域の変異を検出するキットであるフィノス LiPA Rif TB を用いて，RFP 耐性 *M. kansasii* の反応を検討した。しかし *M. kansasii* に対してプローブの検出が全く認められなかった (Fig. 2)。Fig. 1で示したシークエンス解析結果から，*M. kansasii* は結核菌と同じアミノ酸配列を有する *rpoB* 領域をもつが，塩基配列では結核菌と違う構造をもつため，*M. kansasii* は同キットでは反応しなかったものと思われる。

M. kansasii の野生株は基本的に RFP 感受性であり，治療中に耐性を獲得するといわれている[4)11)]。今回の検証では全 *M. kansasii* に占める RFP 耐性菌の割合は314株中3株 (0.96%) であり，1989年から1992年の間に実施された米国テキサス州での大規模な疫学調査 (464株) で RFP 耐性 *M. kansasii* の占める割合が17株 (4%)[4] だった結果と比較しても耐性率はかなり低い。実施期間の違いや地域差を考慮する必要があるが，薬剤感受性試験をルーチンとしてすべての *M. kansasii* に実施することは非効率と考えられる結果となった。今後コスト面での対応も含めて考えていく必要があろう。

今回 RFP 耐性 *M. kansasii* は，結核菌群と同じ hot spot 領域に *rpoB* 遺伝子変異を認めたが，RFP 感受性 *M. kansasii* は *rpoB* 遺伝子変異を認めなかった。これらのことから，RFP 耐性 *M. kansasii* と *rpoB* 遺伝子変異との間に強い関連性が証明された。

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Original Article

DETECTION OF *rpoB* MUTATIONS IN RIFAMPICIN-RESISTANT *MYCOBACTERIUM KANSASII*

[1]Shiomi YOSHIDA, [1]Katsuhiro SUZUKI, [1]Kazunari TSUYUGUCHI, [4]Tomotada IWAMOTO, [2]Motohisa TOMITA, [1]Masaji OKADA, [3]Mitsunori SAKATANI

Abstract [Purpose] To detect rifampicin-resistant mutations in *Mycobacterium kansasii* (*M. kansasii*).

[Methods] We examined the *M. kansasii* isolates from sputum of patients at National Hospital Organization Kinki-chuo Chest Medical Center from January 1, 2001 to November 30, 2005 using drug-susceptibility testing, and analyzed 69-bp fragment of *rpoB* gene in rifampicin-resistant strains.

[Results] Three strains from 314 isolates were determined as rifampicin resistant using drug-susceptibility testing. Those strains showed a rise in minimum inhibitory concentration (MIC), and had the mutations in *rpoB* gene. These point mutations in codons 513 and 516 were common mutations found in rifampicin-resistant clinical isolates of *M.tuberculosis*.

[Discussion] We verified the association between *rpoB* gene mutations and rifampicin resistance in *M. kansasii*.

Key words: *Mycobacterium kansasii*, Rifampicin-resistance, *rpoB* mutations, Drug-susceptibility test

[1]Clinical Research Center, [2]Department of Clinical Laboratory, [3]Department of Respiratory Medicine, National Hospital Organization Kinki-chuo Chest Medical Center, [4]Kobe Institute of Health

Correspondence to: Shiomi Yoshida, Clinical Research Center, National Hospital Organization Kinki-chuo Chest Medical Center, 1180 Nagasone-cho, Kita-ku, Sakai-shi, Osaka 591–8555 Japan. (E-mail: dustin@kch.hosp.go.jp)

OPC-67683, a Nitro-Dihydro-Imidazooxazole Derivative with Promising Action against Tuberculosis In Vitro and In Mice

Makoto Matsumoto[1*], Hiroyuki Hashizume[1], Tatsuo Tomishige[1], Masanori Kawasaki[1], Hidetsugu Tsubouchi[2], Hirofumi Sasaki[2], Yoshihiko Shimokawa[3], Makoto Komatsu[2]

1 Microbiological Research Institute, Otsuka Pharmaceutical, Tokushima, Japan, **2** Medicinal Chemistry Research Institute, Otsuka Pharmaceutical, Tokushima, Japan, **3** Tokushima Research Institute, Otsuka Pharmaceutical, Tokushima, Japan

Funding: Otsuka Pharmaceutical was the sole financial supporter of the studies. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: All of the authors are working as scientists for Otsuka Pharmaceutical, the originator and owner of OPC-67683 and sole financial supporter of the studies. However, the company is not publicly traded, and none of the authors have or are expected to have stock options.

Academic Editor: Philip Hopewell, University of California San Francisco, United States of America

Citation: Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, et al. (2006) OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. PLoS Med 3(11): e466. doi:10.1371/journal.pmed.0030466

Received: February 20, 2006
Accepted: September 20, 2006
Published: November 28, 2006

Abbreviations: BRM, bacterial reverse mutation; CFU, colony-forming unit; DMSO, dimethylsulfoxide; EB, ethambutol; HPLC, high-performance liquid chromatography; ICR, Institute of Cancer Research; INH, isoniazid; LTBI, latent tubercle bacilli infection; MDR-TB, multidrug-resistant tuberculosis; MIC, minimum inhibitory concentration; PZA, pyrazinamide; RFP, rifampicin; SM, streptomycin; TB, tuberculosis

* To whom correspondence should be addressed. E-mail: m_matsumoto@research.otsuka.co.jp

ABSTRACT

Background

Tuberculosis (TB) is still a leading cause of death worldwide. Almost a third of the world's population is infected with TB bacilli, and each year approximately 8 million people develop active TB and 2 million die as a result. Today's TB treatment, which dates back to the 1970s, is long and burdensome, requiring at least 6 mo of multidrug chemotherapy. The situation is further compounded by the emergence of multidrug-resistant TB (MDR-TB) and by the infection's lethal synergy with HIV/AIDS. Global health and philanthropic organizations are now pleading for new drug interventions that can address these unmet needs in TB treatment.

Methods and Findings

Here we report OPC-67683, a nitro-dihydro-imidazooxazole derivative that was screened to help combat the unmet needs in TB treatment. The compound is a mycolic acid biosynthesis inhibitor found to be free of mutagenicity and to possess highly potent activity against TB, including MDR-TB, as shown by its exceptionally low minimum inhibitory concentration (MIC) range of 0.006–0.024 μg/ml in vitro and highly effective therapeutic activity at low doses in vivo. Additionally, the results of the post-antibiotic effect of OPC-67683 on intracellular *Mycobacterium tuberculosis* showed the agent to be highly and dose-dependently active also against intracellular *M. tuberculosis* H37Rv after a 4-h pulsed exposure, and this activity at a concentration of 0.1 μg/ml was similar to that of the first-line drug rifampicin (RFP) at a concentration of 3 μg/ml. The combination of OPC-67683 with RFP and pyrazinamide (PZA) exhibited a remarkably quicker eradication (by at least 2 mo) of viable TB bacilli in the lung in comparison with the standard regimen consisting of RFP, isoniazid (INH), ethambutol (EB), and PZA. Furthermore, OPC-67683 was not affected by nor did it affect the activity of liver microsome enzymes, suggesting the possibility for OPC-67683 to be used in combination with drugs, including anti-retrovirals, that induce or are metabolized by cytochrome P450 enzymes.

Conclusions

We concluded that based on these properties OPC-67683 has the potential to be used as a TB drug to help combat the unmet needs in TB treatment.

The Editors' Summary of this article follows the references.

Introduction

Tuberculosis (TB) is still a leading cause of death worldwide [1]. Almost a third of the world's population is infected with TB bacilli, and each year approximately 8 million people develop active TB and 2 million die as a result [2]. Today's TB treatment, which dates back to the 1970s, is long and burdensome, requiring at least 6 mo of multidrug chemotherapy, typically consisting of rifampicin (RFP), isoniazid (INH), ethambutol (EB), and pyrazinamide (PZA) given under clinically observed conditions. The situation is further complicated by the emergence of multidrug-resistant TB (MDR-TB) and by the infection's lethal synergy with HIV/AIDS [3–6]. Patients with MDR-TB must be treated with a combination containing second-line drugs that are less effective, more expensive, and more toxic. TB's lethal synergy with HIV/AIDS puts HIV-positive individuals with latent tubercle bacilli infection (LTBI) at a 30× to 50× greater risk of developing active TB, giving rise to TB as the number one killer among patients with AIDS [6].

The pharmaceutical industry, however, has generally shown little interest in developing new, more effective drugs to address these needs, and, as a result, no new anti-TB agent with a novel mechanism of action has been launched since the introduction of RFP in 1966. Consequently, global health and philanthropic organizations are now pleading for new chemotherapy interventions that can shorten the total duration of therapy, provide improved efficacy against MDR-TB, safely treat patients co-infected with HIV/AIDS, and target LTBI [6,7].

We initiated a program to screen for potent anti-TB agents that have a new structure and mechanism able to inhibit the biosynthesis of mycolic acid, and found nitro-dihydro-imidazooxazole derivatives to exhibit such activity. Nitro-heterocyclic compounds, including various 5- and 2-nitro-imidazoles and 5-nitrofurans, are known to be effective against a variety of protozoan and bacterial infections in humans and animals [8]. These compounds, however, are also known to commonly possess mutagenicity. CGI-17341 (Figure 1), a nitroimidazooxazole derivative, has been reported to have anti-tubercular activity [9,10], but the compound was not developed because of its mutagenic properties. We focused our search on new nitro-dihydro-imidazooxazoles with anti-tubercular activity that had no mutagenicity by performing the bacterial reverse mutation (BRM) test [11]. About 95% of the compounds we screened earlier that had mono- or di-alkyl substituents at 2-position were mutagenic. However, after introducing heteroatoms to the substituent, we were able to successfully decrease the mutagenicity rate to 16%. Among the non-mutagenic derivatives, we found OPC-67683 to have potent anti-TB activity. We then further evaluated OPC-67683 to determine whether the compound could help address the unmet needs of TB treatment.

Methods

Culture Medium

Cultures of *Mycobacterium tuberculosis* and *M. bovis* BCG were grown in Middlebrook 7H9 broth (BBL, http://www.bd.com) and Middlebrook 7H11 agar medium (BBL), respectively. Both types of media were prepared according to the manufacturer's directions.

Drug Preparation for In Vitro Studies

OPC-67683, PA-824, and CGI-17341 were synthesized at Otsuka Pharmaceutical (http://www.otsuka.global.com); RFP, INH, EB, streptomycin (SM), and PZA were purchased from Sigma (http://www.sigmaaldrich.com). OPC-67683, RFP, INH, PZA, and PA-824 were each dissolved in dimethylsulfoxide (DMSO), and the solutions were diluted serially with DMSO in 2-fold dilutions to desired concentrations. EB and SM were dissolved in distilled water, and the solutions were serially diluted with distilled water in 2-fold dilutions to desired concentrations.

Drug Preparation for In Vivo Studies

OPC-67683, PA-824, RFP, INH, EB, and PZA were each pestled in a mortar and dissolved or suspended in 5% gum arabic solution using an ultrasonic generator. Two-fold dilutions were then conducted using 5% gum arabic solution to adjust to the desired concentrations.

Strains

M. tuberculosis ATCC 25618 (H37Rv), *M. tuberculosis* ATCC 35838 (H37Rv-R-R), *M. tuberculosis* ATCC 35822 (H37Rv-H-R), *M. tuberculosis* ATCC 35837 (H37Rv-E-R), *M. tuberculosis* ATCC 35820 (H37Rv-S-R), *M. tuberculosis* ATCC 35801 (Erdman), and *M. tuberculosis* ATCC 35812 (Kurono) were purchased from American Type Culture Collection (http://www.atcc.org). *M. bovis* IID 982 (BCG Tokyo) was purchased from the Institute of Medical Science, University of Tokyo. A total of 67 *M. tuberculosis* strains used in this study were isolated in Japan, Myanmar, Thailand, Cambodia, Indonesia, Vietnam, and China.

BRM Test

The BRM test was performed in accordance with OECD Guideline 471 using *Salmonella tyiphimurium* TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2 *uvrA* [11]. Each bacterial strain was pre-cultured at 37 °C for 18 h using a nutrient broth (Nissui Pharmaceutical; http://www.nissui-pharm.co.jp/index__e.html). After adjustment to 2.4 at OD660 nm, each bacterial suspension was added to a test tube containing the designated compound in the absence or presence of rat liver microsome (S9) mix. After a 20-min incubation at 37 °C, top agar was added to each test tube and the contents were poured into minimum essential medium (Oriental Yeast; http://www.oyc.co.jp/e/index.htm). The number of revertants was counted 48 h after incubation at 37 °C.

Susceptibility Testing

Susceptibility testing was performed using a procedure previously reported [12,13]. Bacteria stocks preserved in a deep freezer were each dissolved and adjusted to approximately 10^6 colony-forming units (CFU)/ml. Drug-containing plates were inoculated with the designated bacterial suspension to approximately 10^6 CFU/ml using a multipoint inoculator (Sakuma Seisakusho; http://homepage1.nifty.com/sakuma2000). Each plate was incubated at 37 °C for 14 d and analyzed to determine the minimum inhibitory concentration (MIC). The MIC was expressed as the lowest concentration that inhibited visible growth of organism on the agar medium after incubation.

For the evaluation of susceptibility against clinically isolated strains, resistance was determined based on the following criteria recommended by the National Committee

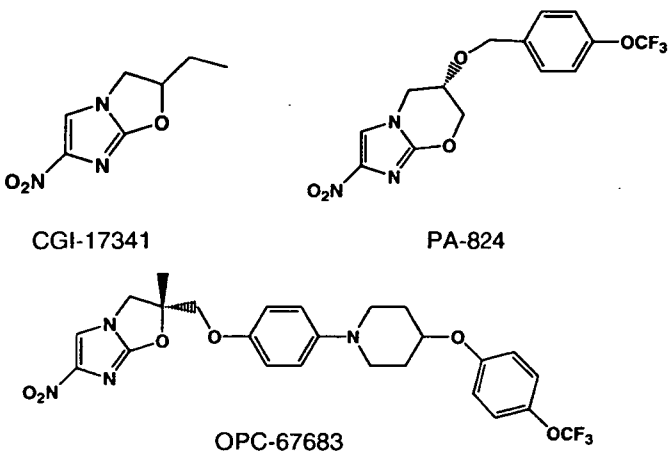


Figure 1. Structure of CGI-17341, PA-824, and OPC-67683
OPC-67683: (*R*)-2-methyl-6-nitro-2-{4-[4-(4-trifluoromethoxyphenoxy)piperidin-1-yl]phenoxymethyl}-2,3-dihydroimidazo[2,1-*b*]oxazole.
doi:10.1371/journal.pmed.0030466.g001

for Clinical Laboratory Standards [14]: 1.0 μg/ml for RFP, 1.0 μg/ml for INH, 7.5 μg/ml for EB, and 10 μg/ml for SM. We calculated the concentrations at which 90% of the susceptible strains were inhibited (MIC_{90}) and the 95% confidence intervals using the probit method.

Inhibitory Activity against Mycolic Acid Biosynthesis

M. bovis BCG cell culture was apportioned to each assay tube at a volume of 0.98 ml, and then 0.01 ml of the test sample solution or DMSO (vehicle control) was added. Then, 0.01 ml of 2-^{14}C acetic acid sodium salt was added to each tube at 1 mCi/tube (37 Bq/tube), followed by incubation at 37 °C for 60 min. The ^{14}C-labeled cells were harvested by centrifugation at 2,000 × g for 10 min and hydrolyzed by 2 ml of 10% potassium hydroxide/methanol (20% potassium hydroxide:methanol = 1:1, vol/vol) at 37 °C for 1 h. After incubation, 1 ml of 6 M hydrochloric acid was added and mixed gently. Then, 5 ml of n-hexane was added, followed by extraction by shaking for 20 min. Separating upper-phase centrifugation (1,000 × g for 5 min) was then performed, and 4 ml of the upper hexane phase was removed and transferred to another tube and dried at 100 °C. For methyl esterization, 1 ml of benzene-methanol-concentrated sulfuric acid (10:20:1, vol/vol/vol) was added and incubated at 100 °C for 1 h for drying. Then, 0.2 ml of n-hexane was added and mixed to extract ^{14}C-labeled fatty acid and mycolic acid. The extracted fatty acid and the mycolic acid subclasses were separated onto a thin-layer plate of Silicagel 60 F254 (thin-layer chromatography plate, Merck Japan; http://www.merck.co.jp/eng/index.html). 0.01 ml of extracted hexane phase was applied to the plate and allowed to develop to a diameter of 4 cm in the first solvent (heptan–diethylether–acetic acid [94:5:1, vol/vol/vol]) and 8 cm in the second solvent (petroleum ether–acetic acid [98:2, vol/vol]). Three thin-layer chromatography plates were fixed with an imaging plate (BAS-SR, Fujifilm; http://www.fujifilm.com) and analyzed by the following procedures: ^{14}C-labeled fatty acid and mycolic acid were detected using a BAS-2500 imaging system (Fujifilm). The radioactivity of each mycolic acid subclass was calculated as photo-stimulated luminescence using Image Gauge software (Version 2.54).

Statistical analysis was conducted, using SAS software (R.8.1, SAS Institute; http://www.sas.com), on the values of percent of control that were calculated automatically using Image Gauge software (Version 2.54) based on the result of each photo-stimulated luminescence. The significance level of the test was set at 5%. IC_{50} values (concentration required to inhibit by 50%) and 95% confidence intervals were calculated by linear regression analysis with logarithmic transformed concentrations.

Analysis of Metabolites Produced after Mixing OPC-67683 and *M. bovis* BCG Tokyo

15 μl of ^{14}C OPC-67683 (0.5 mg/ml:1 μCi/μl) was added to 585 μl of 7H9/TN-ADC broth or bacterial culture and incubated for 48 h. After incubation, a 2-fold volume of acetonitrile was added and mixed well. The lysate was centrifuged for 5 min at 15,000 rpm, and the supernatant was analyzed using high-performance liquid chromatography (HPLC) with flow scintillation analyzer to determine the metabolite pattern. In a parallel experiment, 0.1 ml of the supernatant was added to the vial containing 5 ml of Scintillation Cocktail (Ultima Gold, Perkin Elmer; http://www.perkinelmer.com). The pellet was suspended in 600 μl of 2 M sodium hydroxide and incubated for 1 h at 60 °C, and 0.1 ml of the suspension was added to the vial containing the Scintillation Cocktail. These samples were measured using a Scintillation Counter (LS5000CE, Beckman; http://www.beckmancoulter.com) to confirm the existence of covalently binding radioactive molecules.

Determination of the Structure of Metabolite Produced after Mixing OPC-67683 and *M. bovis* BCG Tokyo

75 μl of OPC-67683 (0.5 mg/ml) was added to 2,925 μl of 7H9/TN-ADC broth or *M. bovis* BCG Tokyo bacterial culture and incubated for 72 h. After incubation, a 2-fold volume of acetonitrile was added and mixed well. The lysate was centrifuged for 5 min at 15,000 rpm, and the supernatant was then analyzed using LC-MS/MS to determine the structure of the detected metabolite produced by mixing OPC-67683 with *M. bovis* BCG Tokyo. The identified metabolite was synthesized at Otsuka Pharmaceutical, and the fragment pattern of the metabolite was then compared with that of another compound newly synthesized based on the predicted structure.

Activity against Intracellular Mycobacteria

Human THP-1 monocytic cells were differentiated into macrophages by treatment with 100 ng/ml phorbol 12-myristae 13-acetate (PMA) in RPMI-1640 medium and were distributed at a portion of 1×10^6/ml after a 2-d incubation. The differentiated macrophages were then inoculated with 6.88 $\log_{10}$ CFU of *M. tuberculosis* H37Rv for 4 h, washed twice with the medium to roughly remove the non-infecting bacteria, and then treated with 20 μg/ml SM for 20 h to kill the remaining viable extracellular bacteria. The starting CFU count in the cells was 6.42 $\log_{10}$ CFU. The cells were subsequently treated with the designated test compound for 4 h and were then washed twice with fresh medium to remove the added test compound. After an additional 68-h culture, the cells were lysed using 0.1% SDS, and the viable bacteria were counted in 7H11 agar plates to determine the potency against intracellular mycobacteria.

Plasma Levels in an Experimental Mouse Model of TB

Mice were anesthetized by an intramuscular administration with a 0.05-ml solution containing ketamine and xylazine (Ketalar 50 [Sankyo; http://www.sankyo.co.jp/english]/ Serakutaru 2% [Bayer; http://www.bayer.com])/sterile physiological saline solution = 8:3:9), infected by an intratracheal inoculation with a 0.05-ml cell suspension (1,010 CFU) of *M. tuberculosis* Kurono using feeding needle and micro-syringe, and housed for 28 d prior to the initiation of administration. The designated compound dissolved or suspended in 5% gum arabic was then administered orally. Blood samples (approximately 1 ml) at each time-point were collected into a heparinized syringe from the abdominal post cava under ether anesthesia. The blood samples were then centrifuged (3,000 rpm, at 5 °C) to extract the plasma. The plasma (0.1 ml) was mixed with acetonitrile (0.2 ml) for RFP and with ethanol (0.3 ml) for INH, EB, and PZA. For OPC-67683, the plasma obtained was filtered through a 0.22-μm filter, and then 0.1 ml of the filtered plasma was mixed with 0.5 ml of 0.5 M carbonate buffer (pH 10) and 5 ml of diethyl ether. After shaking for 10 min, the organic layer (4 ml) was dried using nitrogen gas at 40 °C and dissolved with 0.2 ml of methanol/ water/formic acid (50/50/0.1). The samples were analyzed using HPLC and high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS).

Therapeutic Efficacy

For evaluation of the therapeutic efficacy of OPC-67683, we designed three experiments that used various mouse models of TB, as described below. In each experiment, the designated compound dissolved or suspended in 5% gum arabic was administered orally once daily. 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. After spreading the homogenate solution, all plates were incubated at 37 °C and counted for formed colonies after 14 d.

Therapeutic efficacy in an experimental mouse model of chronic TB. In order to examine the therapeutic efficacy of OPC-67683 and to determine the therapeutic dose range, an experimental mouse model of chronic TB was established by inoculating Institute of Cancer Research (ICR) mice with *M. tuberculosis* Kurono through the caudal vein and allowing the infection to develop for 28 d. OPC-67683, RFP, INH, EB, SM, or PZA was then administered once daily for 28 d to examine the change in viable bacterial count in the lung. ICR mice were inoculated intravenously with 8.6×10^4 CFU of *M. tuberculosis* Kurono. After a 28-d period, the mice were assigned to groups ($n = 5$/group) using a stratified randomization method based on the body weight of each infected mouse. The test compounds were then administered orally once daily for 28 d (OPC-67683: 40 to 0.156 mg/kg, RFP: 20 to 1.25 mg/kg, INH: 20 to 1.25 mg/kg, EB: 160 to 20 mg/kg, SM: 160 to 20 mg/kg, PZA: 320 to 40 mg/kg, and PA-824: 40 to 1.25 mg/kg [2-fold dilutions]). CFU counts were performed as described above. All lungs were homogenized with 5 ml of sterile distilled water.

Statistical analysis was conducted using SAS software (R.8.1) on the number of viable bacteria in the lung of mice surviving until necropsy on the 57th day after inoculation, and on the number at the start of the treatment, which was 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.

Therapeutic efficacy in an experimental TB model using immunocompromised mice. To examine whether immunity relates to the mechanism of action in vivo, we performed experiments using BALB/c nude mice, which lack both conventional $CD4^+$ and $CD8^+$ T cells. The anti-tubercular activity of OPC-67683 in nude mice was compared with that in immunocompetent mice. BALB/c nude mice and BALB/c mice were inoculated intravenously with 2.04×10^4 CFU of *M. tuberculosis* Kurono. 1 d after inoculation, the mice were assigned to groups ($n = 5$/group) using a stratified randomization method based on the body weight of each infected mouse. OPC-67683 was then administered orally once daily for 10 d (OPC-67683: 10 to 0.313 mg/kg [2-fold dilutions]). CFU counts were performed as described above. All lungs were homogenized with 5 ml of sterile distilled water.

Therapeutic efficacy in combination with conventionally used drugs. A new regimen that included OPC-67683 was evaluated and compared with a global standard regimen to determine the best regimen for reducing the treatment duration in an experimental mouse model of chronic TB. ICR mice were inoculated intratracheally under anesthesia with 855 CFU of *M. tuberculosis* Kurono, and left for 28 d to allow the animals to develop chronic TB. Grouping ($n = 6$/group) was conducted by a stratified randomization method based on the body weight of each infected mouse. The test regimens were then administered orally for 2 mo in the combination of OPC-67683, RFP, and PZA, or RFP, INH, EB, and PZA as an intensive treatment, and for an additional 2 mo in the combination of OPC-67683 and RFP or 4 mo in the combination of RFP and INH as a maintenance treatment. The doses used in this experiment provided plasma levels in mice similar to those seen at the standard doses used in humans: for RFP, we used 5 mg/kg; for INH, 10 mg/kg; for EB, 100 mg/kg; and for PZA, 100 mg/kg. We set the dose for OPC-67683 at 2.5 mg/kg.

Necropsy was performed on days 29, 57, 85, 113, 141, 169, and 177 relative to the inoculation for the standard regimen and vehicle control groups and on days 29, 57, 85, 113, and 141 for the new-regimen groups. A lung homogenate for each mouse from a drug-treated group was prepared by pestling the lung evenly with a glass homogenizer after adding to the excised lungs 5 ml of sterilized distilled water on day 29 and 2 ml of sterilized distilled water on the day of necropsy. Lung homogenates for all vehicle control groups were prepared by pestling the lung evenly with a glass homogenizer after adding 5 ml of sterilized distilled water to the excised lungs. Smear plates of lung homogenate samples from the groups after 2–6

Table 1. Bacterial Reverse Mutation Test for OPC-67683

Bacterial Strain	S9 mix	Compound	Revertants/Plate					
			5,000	2,500	1,250	625	312.5	0 μg/plate
S. typhimurium TA98	−	OPC-67683 AF-2(0.1 μg/plate)	31	37	34	29	29	29 473
	+	OPC-67683 2AA(0.5 μg/plate)	35	31	30	31	31	36 92
S. typhimurium TA100	−	OPC-67683 AF-2(0.01 μg/plate)	94	90	87	77	85	98 547
	+	OPC-67683 2AA(1 μg/plate)	119	112	107	108	116	108 1103
S. typhimurium TA1535	−	OPC-67683 NaN_3(0.5 μg/plate)	6	7	6	9	6	6 174
	+	OPC-67683 2AA(2 μg/plate)	9	8	5	6	5	6 188
S. typhimurium TA1537	−	OPC-67683 ACR(80 μg/plate)	63	61	54	62	60	64 953
	+	OPC-67683 2AA(2 μg/plate)	74	63	66	72	73	81 238
E. coli WP2 uvr A	−	OPC-67683 AF-2(0.01 μg/plate)	24	24	20	25	23	30 225
	+	OPC-67683 2AA(10 μg/plate)	29	23	25	20	23	33 1122

AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; 2AA, 2-aminoanthracene; NaN_3, sodium azide; ACR, 9-aminoacridine.
doi:10.1371/journal.pmed.0030466.t001

mo of treatment were prepared by spreading all of the lung homogenate on 7H11 agar plates.

Statistical analysis was conducted using SAS software (R.8.1) on the viable bacteria number in the lungs of mice surviving until necropsy after the inoculation. The significance level of the test was set at 5%. The viable bacterial count in the lungs of mice anatomized at days 57, 85, 113, and 141 were log-transformed for comparing the new regimen with the standard regimen using the two-tailed Dunnett's test. The mean values and 95% confidence intervals were calculated for evaluating the new regimen.

In Vitro Metabolism of OPC-67683 in Human and Animal Liver Microsomes

The study was undertaken to investigate the metabolites produced by the metabolic reactions of OPC-67683 using human, rat, mouse, dog, rabbit, and monkey liver microsomes. Pooled human liver microsomes (20 mg/ml) from ten donors were prepared at the Biomedical Research Institute, Human and Animal Bridge Discussion Group (Chiba, Japan) [15]. Human liver samples were legally procured from the National Disease Research Interchange (http://www.ndriresource.org/) through the international partnership with the Human and Animal Bridge Discussion Group. The study was conducted in accordance with the Declaration of Helsinki.

The incubation mixtures contained 100 mM phosphate buffer (pH 7.4), 100 μM OPC-67683, 2.5 mM β-NADPH, 2.5 mM β-NADH, and 1 mg/ml microsomal protein in a final incubation volume of 0.5 ml. OPC-67683 was dissolved in DMSO, and the concentration of the organic solvent was 1% (v/v) in the reaction system. The reactions were performed in duplicate in a shaking water bath at 37 °C for 2 h. The incubation mixtures were extracted with acetonitrile and ethyl acetate, and the samples were analyzed by HPLC and LC-ESI-MS/MS.

Effect of OPC-67683 on Cytochrome P450–Mediated Reactions in Human Liver Microsomes

7-ethoxyresorufin *O*-deethylase activity by CYP1A1/2, coumarin 7-hydroxylase activity by CYP2A6, 7-benzyloxyresorufin *O*-debenzylase activity by CYP2B6, tolbutamide methylhydroxylase activity by CYP2C8/9, S-mephenytoin 4′-hydroxylase activity by CYP2C19, bufuralol 1′-hydroxylase activity by CYP2D6, chlorzoxazone 6-hydroxylase activity by CYP2E1, and testosterone 6β-hydroxylase and nifedipine oxidized activities by CYP3A4 were determined as previously reported [16].

Standard incubation mixtures of 0.5 ml contained microsomal protein (0.1–0.5 mg), 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, NADPH-generating system (2.5 mM β-NADP, 25 mM glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and 10 mM magnesium chloride), and substrates with or without OPC-67683. OPC-67683 was dissolved in DMSO and added to incubations at a volume of 5 μl. Substrates were dissolved in the following solvents: 7-ethoxyresorufin and 7-benzyloxyresorufin in DMSO; coumarin, bufuralol, and nifedipine in ethanol; tolbutamide, *S*-mephenytoin and testosterone in methanol; and chlorzoxazone in 1% (w/v) aqueous solution. The substrate solutions were added to incubations at a volume of 5 μl. The enzyme incubations were carried out in duplicate, and formations of metabolites were determined by HPLC.

Assay methods were validated in this study. The calibration curves were established for resorufin (0.2–200 nM, $r = 0.9996$), 7-hydroxycoumarin (0.05–5 μM, $r = 0.9998$), 4-hydroxytolbutamide (0.05–10 μM, $r = 0.9998$), 4-hydroxymephenytoin (0.025–5 μM, $r = 0.9996$), 1′-hydroxybufuralol (0.025–5 μM, $r = 0.9995$), 6-hydroxychlorzoxazone (0.25–100 μM, $r = 0.9994$), 6β-hydroxytestosterone (0.03–30 μM, $r = 0.9994$), and oxidized nifedipine (0.1–25 μM, $r = 0.9998$).

7-ethoxyresorufin (0.5 μM), coumarin (2 μM), 7-benzyloxyresorufin (1.5 μM), tolbutamide (400 μM), *S*-mephenytoin (100 μM), bufuralol (20 μM), chlorzoxazone (100 μM), testosterone (100 μM), and nifedipine (50 μM) were selected as the concentrations of the substrates for the determination of residual activity in the presence of OPC-67683 (1–100 μM). The concentrations of the substrates were approximately the K_m values for the enzymes as previously reported [17]. Selective Cytochrome P450 inhibitors were used in this study to confirm the validity of the assays. 7,8-benzoflavone [18], furafylline [19], orphenadrine [20], quercetin [21], sulfaphenazole [22], tranylcypromine [23], quinidine [24], diethyldithiocarbamate [25], and ketoconazole [26], which are inhibitors of CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, respectively, inhibited the respective enzyme

Table 2. In Vitro Anti-Mycobacterial Activity of OPC-67683 Compared with RFP, INH, EB, SM, CGI-17341, and PA-824

Type Strain	MIC (μg/ml)						
	OPC-67683	RFP	INH	EB	SM	CGI-17341	PA-824
M. tuberculosis ATCC 25618 (H37Rv)	0.012	0.78	0.1	1.56	1.56	0.2	0.2
M. tuberculosis ATCC 35838 (H37Rv-R-R)	0.006	>100	0.1	1.56	0.78	0.05	0.1
M. tuberculosis ATCC 35822 (H37Rv-H-R)	0.012	0.39	>100	3.13	0.78	0.2	0.05
M. tuberculosis ATCC 35837 (H37Rv-E-R)	0.012	0.2	0.2	50	0.78	0.2	0.2
M. tuberculosis ATCC 35820 (H37Rv-S-R)	0.012	0.78	0.1	3.13	>100	0.2	0.2
M. tuberculosis ATCC 35812 (Kurono)	0.012	0.39	0.1	3.13	0.78	0.2	0.2

Susceptibility of OPC-67683 against standard *M. tuberculosis*.
doi:10.1371/journal.pmed.0030466.t002

activities. Diethyldithiocarbamate is also known to be a specific inhibitor of CYP2A6 [18], and the present study confirmed the potent inhibitory capability of this compound on CYP2A6-mediated metabolism.

Other Information

The care and handling of the animals was in accordance with "Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co., Ltd." The aspects of experiments related to biosafety were performed according to standards set forth in "Biosafety manuals in Microbiological Research Institute and 3rd Institute of New Drug Discovery, Otsuka Pharmaceutical Co., Ltd."

Results

BRM Test

The mutagenic potential of OPC-67683 was evaluated in the absence and presence of S9 mix using the BRM test in accordance with OECD Guideline 471. As shown in Table 1, OPC-67683 did not show mutagenicity.

Susceptibility Testing

The MICs against standard strains are shown in Table 2. At concentrations ranging from 0.006 to 0.012 μg/ml, OPC-67683 inhibited the growth of both drug-susceptible and drug-resistant *M. tuberculosis*. The MICs of OPC-67683 were, respectively, four to 64, two to 32, 128 to 256, 64 to 512, eight to 16, and four to 16 times lower than those of RFP, INH, EB, SM, CGI-17341, and PA-824. These results indicate that OPC-67683 possesses the most potent anti-mycobacterial activity against both drug-susceptible and drug-resistant strains.

Table 3. MIC_{90} of OPC-67683 against Drug-Susceptible and Drug-Resistant *M. tuberculosis*

Organism Group (Number of Strains)	MIC (μg/ml)	
	MIC_{90}	95% Confidence Intervals
RFP-susceptible *M. tuberculosis* (31)	0.01248	0.01097–0.01535
RFP-resistant *M. tuberculosis* (36)	0.01221	0.01050–0.01583
INH-susceptible *M. tuberculosis* (31)	0.01194	0.01054–0.01452
INH-resistant *M. tuberculosis* (36)	0.01279	0.01094–0.01679
EB-susceptible *M. tuberculosis* (56)	0.01213	0.01081–0.01440
EB-resistant *M. tuberculosis* (11)	0.01341	0.01073–0.02450
SM-susceptible *M. tuberculosis* (49)	0.01203	0.01077–0.01416
SM-resistant *M. tuberculosis* (18)	0.0134	0.01068–0.02298

Susceptibility of OPC-67683 against 67 strains of clinically isolated *M. tuberculosis*: Resistant strains were selected based on the recommendations of the National Committee For Clinical Laboratory Standards [14] using the following criteria: 1.0 μg/ml for RFP, 1.0 μg/ml for INH, 7.5 μg/ml for EB, and 10 μg/ml for SM. We calculated the concentrations at which 90% (MIC_{90}) of the susceptible strains are inhibited. MIC_{90} and 95% confidence intervals were calculated using the actual data obtained by the probit method.
doi:10.1371/journal.pmed.0030466.t003

The anti-tubercular activity was also evaluated on 67 clinically isolated strains. The MIC_{90} values (range) of OPC-67683, RFP, INH, EB, and SM were, respectively, 0.012 μg/ml (0.006–0.024 μg/ml), 0.288 μg/ml (0.05–0.78 μg/ml), 0.099 μg/ml (0.05–0.78 μg/ml), 3.636 μg/ml (0.78–6.25 μg/ml), and 2.938 μg/ml (0.39–6.25 μg/ml). Based on these results, the MIC_{90} values of OPC-67683 were about 24, eight, 303, and 244 times lower than those of RFP, INH, EB, and SM, respectively. The results of our evaluation indicated that OPC-67683 inhibited the growth of the clinically isolated drug-susceptible *M. tuberculosis* at the same range as on standard strains, and also showed activity against the clinically isolated strains resistant to the currently used anti-TB drugs RFP, INH, EB, or SM. These results indicate that OPC-67683 exhibits anti-mycobacterial activity on both drug-susceptible and drug-resistant strains and that it has no cross-resistance with any of the currently used anti-TB drugs. These data are shown in Table 3.

In addition, the efficacy of OPC-67683 in combination with currently used anti-TB drugs RFP, INH, EB, and SM was examined in vitro using the checkerboard method. These results are shown in Table 4. The results showed OPC-676783 to have no antagonistic activity in combination with any of the drugs tested.

Inhibitory Activity against Mycolic Acid Biosynthesis

^{14}C-labeled fatty acid and mycolic acid were detected using the BAS-2500 imaging system (unpublished data). The percent with respect to the control of each mycolic acid subclass was calculated automatically, and IC_{50} was calculated using SAS software. The results indicated that both OPC-67683 and INH inhibited mycolic acid synthesis, but the manner of action differed between the two compounds: OPC-67683 inhibited the synthesis of methoxy- and keto-mycolic acid, with IC_{50} values of 0.021 to 0.036 μg/ml, but not the synthesis of α-mycolic acid at concentrations up to 0.25 μg/ml, while INH inhibited all mycolic acid subclasses, with IC_{50} values of 0.630 to 1.851 μg/ml. The IC_{50} and 95% confidence interval values are shown in Table 5.

Table 4. In Vitro Synergistic Activity of OPC-67683 and Existing TB Drugs against Clinically Isolated *M. tuberculosis*

Drug Combination	Number of Test Strains for which FIC Index Is:			
	Synergistic	Partially Synergistic	Additive	Indifferent
OPC-67683 and RFP	1 (3.7%)	24 (88.9%)	2 (7.4%)	—
OPC-67683 and INH	—	12 (44.4%)	5 (18.5%)	10 (37.0%)
OPC-67683 and EB	3 (11.1%)	21 (77.8%)	3 (11.1%)	—
OPC-67683 and SM	—	7 (25.9%)	10 (37.0%)	10 (37.0%)

In vitro synergistic activity of OPC-67683 and existing TB drugs against clinically isolated *M. tuberculosis*: The checkerboard procedure was performed based on the MIC values of 27 test strains of clinically isolated *M. tuberculosis* established by the agar dilution method. The level of synergy was determined by calculating the fractional inhibitory concentration (FIC) index based on the following formula: FIC of drug A = MIC of drug A in combination ÷ MIC of drug A alone; FIC of drug B = MIC of drug B in combination ÷ MIC of drug B alone; and FIC index = FIC of drug A + FIC of drug B. Results of FIC index were interpreted as follows: ≤0.5: synergy, >0.5 to 0.75: partial synergy, >0.75 to 1.0: additive effect, >1.0 to 4.0: indifference, and >4.0: antagonism. We calculated the FIC index value for each concentration of two-drug combination and the minimum value was adopted.
doi:10.1371/journal.pmed.0030466.t004

Analysis of Metabolites Produced after Mixing OPC-67683 and *M. bovis* BCG

After mixing OPC-67683 with *M. bovis* BCG Tokyo, we identified only one main metabolite, and this metabolite eluted faster than OPC-67683. No metabolites, however, were observed after mixing OPC-67683 with an experimentally obtained OPC-67683-resistant *M. bovis* BCG Tokyo strain. These results are shown in Figure 2A. The supernatant was analyzed using LC-MS/MS to determine the structure of the identified metabolite. We found the mass number of the identified metabolite to be 490 and predicted this structure to be a desnitro-imidazooxazole. We then synthesized a desnitro-imidazooxazole and performed a product ion scan with the identified metabolite and the newly synthesized compound. We observed product ions in 200, 352, 378, and 406 m/z in each experiment. Structural analysis of the main metabolite indicated that the structure was a desnitro-imidazooxazole possessing the same substituent as that of OPC-67683. The MS spectrum is displayed in Figure 2B.

In addition, when we treated the drug-susceptible strain with the radioactive OPC-67683, none of the radioactivity was recovered after the addition of acetonitrile. About 20% of the total radioactivity was distributed to the cell components, and this phenomenon was not observed with an OPC-67683-resistant strain. These data are shown in Table 6.

Activity against Intracellular Mycobacteria in Human Macrophages

A study was conducted to confirm the post-antibiotic effect of OPC-67683 on intracellular *M. tuberculosis* in THP-1 cells, and the results were compared with RFP, INH, and PA-824. OPC-67683 was shown to be highly active against intracellular *M. tuberculosis* H37Rv after 4-h pulsed exposures in a dose-dependent manner. The data are shown in Figure 3. The intracellular activity of OPC-67683 at a concentration of 0.1 μg/ml was similar to that of RFP of 3 μg/ml, but was superior to INH and PA-824, which both showed poor activity during the 4-h pulsed exposure. These results indicated that even with limited contact with the bacteria within the cells, OPC-67683 might be able to effectively kill the intracellular mycobacteria.

Plasma Levels in an Experimental Mouse Model of TB

As shown in Table 7, OPC-67683 exhibited the lowest plasma concentration but longest half-life among the tested reference drugs. The C_{max} and AUC_t values for RFP, EB, and PZA in mouse plasma at the tested dose were similar to those in human at clinical doses. The C_{max} value for INH in mouse plasma was also similar to that in humans, but the AUC_t in the mouse was lower than that in humans. A comparison of these parameters between mouse and human plasma is summarized in Figure 4C [27–29].

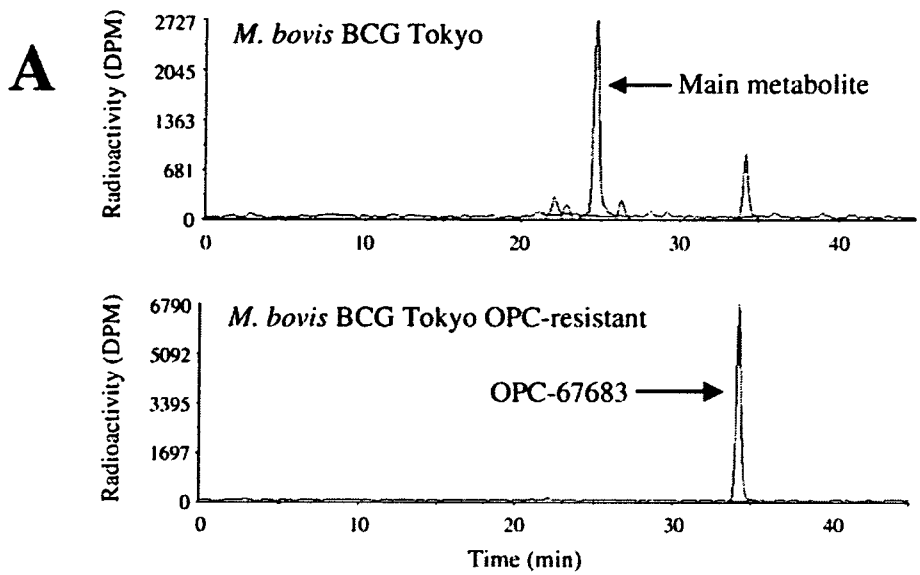
Therapeutic Efficacy

Therapeutic efficacy in an experimental mouse model of chronic TB. The viable bacterial count in the OPC-67683-treated groups decreased dose-dependently, and the therapeutic effects of the compound were observed and compared with those of the reference drugs. The results are shown in Figure 4A and Table S1. The dose groups that showed a significant decrease in pulmonary viable bacterial count when compared with the vehicle control group were

Table 5. IC_{50} of OPC-67683 and INH against Mycolic Acid Synthesis

Compound	Subclass Mycolic Acid and Fatty Acid	IC50 (μg/ml)	95% Confidence Interval (μg/ml)
OPC-67683	Fatty acid	>0.25	—
	α-Mycolic acid	>0.25	—
	Methoxy-mycolic acid	0.036	0.020–0.068
	Keto-mycolic acid	0.021	0.009–0.059
INH	Fatty acid	>4	—
	α-Mycolic acid	1.851	1.109–3.090
	Methoxy-mycolic acid	0.63	0.537–0.738
	Keto-mycolic acid	0.69	0.422–1.129

The IC_{50} (concentration required to inhibit activity by 50%) of OPC-67683 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 OPC-67683 or INH as a reference. ^{14}C-labeled fatty acid and mycolic acid subclasses were detected using thin-layer chromatography (TLC, $n = 3$), and analyzed by BAS-2500 (Fujifilm). The radioactivity of each fatty acid and mycolic acid subclasses was calculated using photo-stimulated luminescence, expressed as the percentage of incorporation in untreated controls, and statistical analysis was conducted by linear regression analysis to calculate IC_{50} values and 95% confidence intervals (significance level: 5%).
doi:10.1371/journal.pmed.0030466.t005



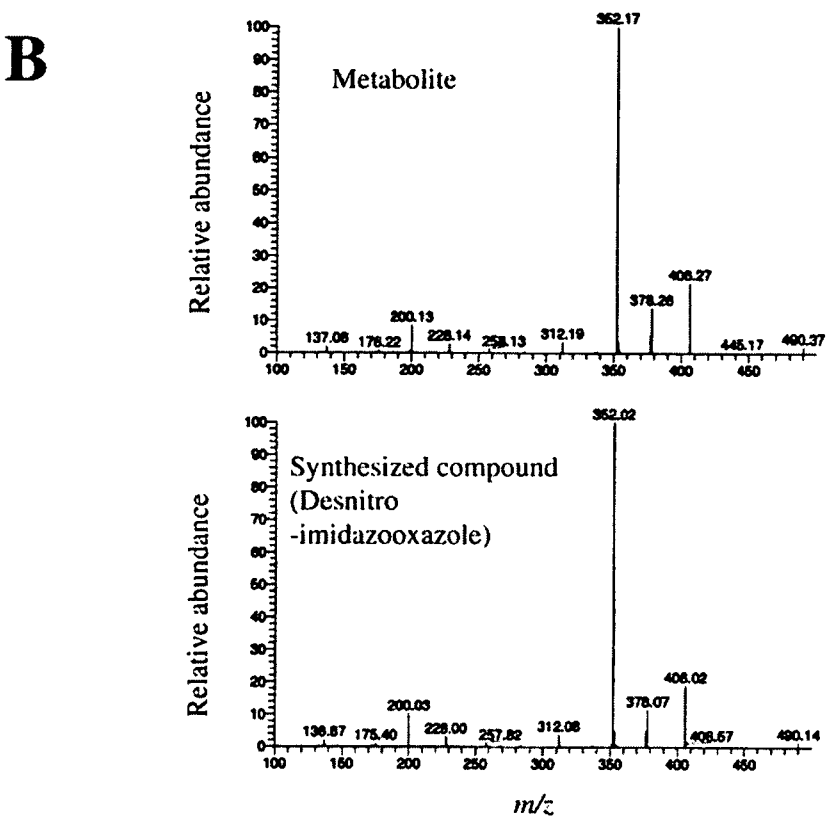


Figure 2. Analysis of Metabolites Produced after Mixing OPC-67683 and *M. bovis* BCG

(A) 15 μl of ^{14}C OPC-67683 (0.5mg/ml: 0.056 μCi/μl) was added to 585 μl of 7H9/TN-ADC broth or bacterial culture and incubated for 48 h. After incubation, a 2-fold volume of acetonitrile was added and mixed well. The lysate was centrifuged for 5 min at 15,000 rpm. The supernatant was analyzed using HPLC with flow scintillation analyzer to determine the metabolite pattern.

(B) The identified metabolite (desnitro-imidazooxazole) was synthesized at Otsuka Pharmaceutical, and the fragment pattern of the metabolite by electrospray ionization mass spectroscopy was then compared with that of another compound newly synthesized based on the predicted structure.

doi:10.1371/journal.pmed.0030466.g002

0.313, 0.625, 1.25, 2.5, 5, 10, 20, and 40 mg/kg for OPC-67683; 3.5, 5, 10, and 20 mg/kg for RFP; 2.5, 5, 10, and 20 mg/kg for INH; 160 mg/kg for EB, 20, 40, 80, and 160 mg/kg for SM; and 80, 160, and 320 mg/kg for PZA.

The doses of OPC-67683, RFP, INH, EB, SM, and PZA that could produce a CFU reduction of at least 95% in this experimental mouse model were 0.625, 3.5, 5, >160, 40, and 160 mg/kg, respectively.

Therapeutic efficacy in an experimental TB model using immunocompromised mice. These results are shown in Figure 4B.

The pulmonary CFU counts of the OPC-67683-treated

Table 6. Analysis of OPC-67683-Susceptible and -Resistant *M. bovis* BCG Using Radio-Labelled OPC-67683

Sample	Total DPM		Percent	
	Supernatant	Pellet	Sample DPM (Supernatant)/ Control DMP (Supernatant)	Sample DPM (Pellet)/ Control DMP (Supernatant)
Control	823295.30	24558.06	100	3
M. bovis BCG Tokyo	678566.21	182886.84	82	22
M. bovis BCG Tokyo OPC-resistant	862893.41	43158.12	105	5

15 μl of ^{14}C OPC-67683 (0.5mg/ml:0.056 μCi/μl) was added to 585 μl of 7H9/TN-ADC broth or bacterial culture and incubated for 48 h. After incubation, a 2-fold volume of acetonitrile was added and mixed well. The lysate was centrifuged for 5 min at 15,000 rpm and 0.1 ml of the supernatant was added to the vial containing 5 ml of Scintillation Cocktail (Ultima Gold, PerkinElmer). The pellet was suspended in 600 μl of 2 M NaOH and incubated for 1 h at 60 °C, and 0.1 ml of the suspension was added to the vial containing 5 ml Scintillation Cocktail. These samples were measured using a Scintillation Counter (LS5000CE, Beckman).
doi:10.1371/journal.pmed.0030466.t006

BALB/c nude mice and immunocompetent mice were reduced dose-dependently, and significant decreases were observed at doses of 0.313, 0.625, 1.25, and 2.5 mg/kg. The efficacy profiles of OPC-67683 were similarly excellent in both types of mice.

Therapeutic efficacy in combination with conventionally used drugs. The eradication rate of a new regimen containing OPC-67683 was compared with that of the standard regimen. The OPC-67683-containing regimen exerted a rapid and consistent reduction during the first 3 mo (Figure 4D). At 3 mo after the start of treatment, only one colony was detected in one of the six animals; at 4 mo, no colonies were detected in any of the six animals. In contrast, at 6 mo for the standard regimen, colonies were detected in four out of five mice. These results suggest that a new regimen containing OPC-67683 could dramatically reduce the treatment duration by at least 2 mo.

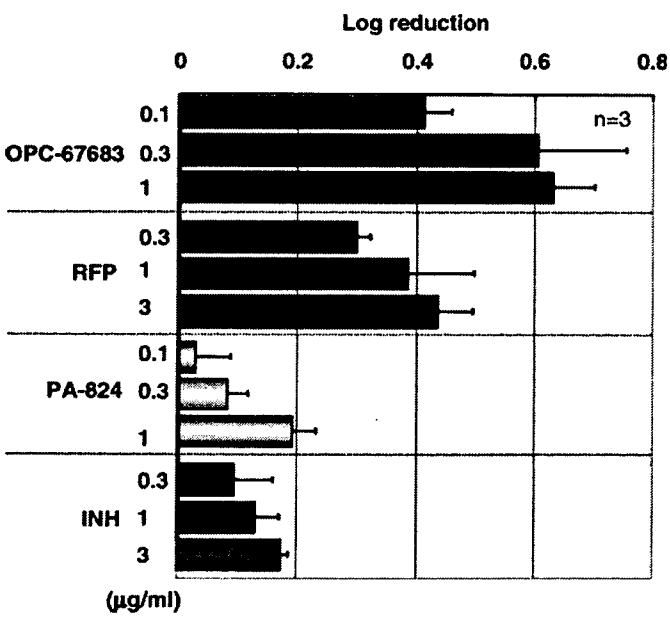


Figure 3. Effect of Pulsed Exposures to OPC-67683, RFP, INH, and PA-824 on the Intracellular Growth of *M. tuberculosis* H37Rv within THP-1 Cells
Infected cells were incubated with the test compound for 4 h, washed, cultured until 68 h at 37 °C, plated on 7H11 agar, and counted for colonies after 16 d of growth at 37 °C. Values represent mean ± S.D ($n = 3$).
doi:10.1371/journal.pmed.0030466.g003

In Vitro Metabolism in Human and Animal Liver Microsomes

The current study was conducted to investigate the metabolites produced by in vitro metabolism of OPC-67683 using human and animal liver microsomes and to investigate the in vitro ability of OPC-67683 to affect the metabolism of substrates for CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. The results are shown in Table 8.

The HPLC and LC-ESI-MS/MS data demonstrated that the major metabolites were hardly detected in the incubation mixture OPC-67683 with human, rat, mouse, dog, rabbit, and monkey liver microsomes. OPC-67683 was stable in the in vitro metabolism of human and animal liver microsomes. These results suggest that OPC-67683 is not metabolized by the CYP enzymes.

OPC-67683 had neither stimulatory nor inhibitory effects on CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 activities at concentrations up to 100 μM, indicating that OPC-67683, at the expected therapeutic concentrations, would not be predicted to cause clinically significant interactions with other CYP-metabolized drugs.

Discussion

With the several disadvantages to the current TB drug regimen, there are a number of expectations for a new anti-TB drug. An ideal new drug should be safe and able to shorten the treatment duration, be effective against MDR-TB, treat TB patients co-infected with HIV, and effectively address LTBI. We have performed our TB research program with these expectations in mind.

To shorten the duration of treatment, we focused our search on finding more powerful anti-TB agents, as history has shown that the introduction of more potent drugs can effectively reduce the required duration of treatment, as was the case with RFP and PZA. For improved efficacy against MDR-TB, we screened for compounds with a new structure and mechanism of action. Furthermore, to target LTBI, we

Table 7. Plasma Concentration of OPC-67683, RFP, INH, EB, and PZA after Oral Administration in Mice Infected with *M. tuberculosis* Kurono

Compound (Dose; mg/kg)	Concentration (μg/ml)											C_{max} (μg/ml)	AUC_t (μg · h/ml)	t_{max} (h)	$t_{1/2}$ (h)
	0.083 h	0.25 h	0.5 h	1 h	2 h	4 h	6 h	8 h	12 h	16 h	24 h				
OPC-67683 (2.5)	N.T.	N.T.	N.T.	0.133 ±0.030	0.193 ±0.040	0.220 ±0.020	0.297 ±0.083	0.167 ±0.028	0.166 ±0.049	N.T.	0.049 ±0.012	0.297	4.13	6	7.6
RFP (5)	N.T.	N.T.	3.33 ±0.87	4.49 ±1.04	4.52 ±1.90	3.82 ±0.70	5.10 ±1.63	3.18 ±0.68	N.T.	0.660 ±0.260	N.T.	5.10	48.2	6	3.4
INH (10)	2.17 ±0.435	3.06 ±0.779	2.28 ±0.390	1.92 ±0.478	0.740 ±0.202	0.253 ±0.057	N.T.	N.D.	N.T.	N.T.	N.T.	3.06	4.55	0.25	1.0
EB (100)	0.055 ±0.049	1.30 ±0.939	3.17 ±0.392	3.51 ±1.13	2.51 ±1.01	1.02 ±0.202	N.T.	0.612 ±0.325	N.T.	N.T.	N.T.	3.51	12.2	1	2.8
PZA (100)	49.6 ±11.2	59.1 ±14.1	63.2 ±28.9	60.2 ±18.7	35.5 ±6.45	18.4 ±3.09	N.T.	0.815 ±0.580	N.T.	N.T.	N.T.	63.2	197	0.5	1.1

Each value represents mean ± SD ($n = 3$).
Each pharmacokinetic parameter was calculated by WINNONLIN (Version 4.1).
N.D., not detected (<0.05 μg/ml for INH); N.T., not tested.
doi:10.1371/journal.pmed.0030466.t007

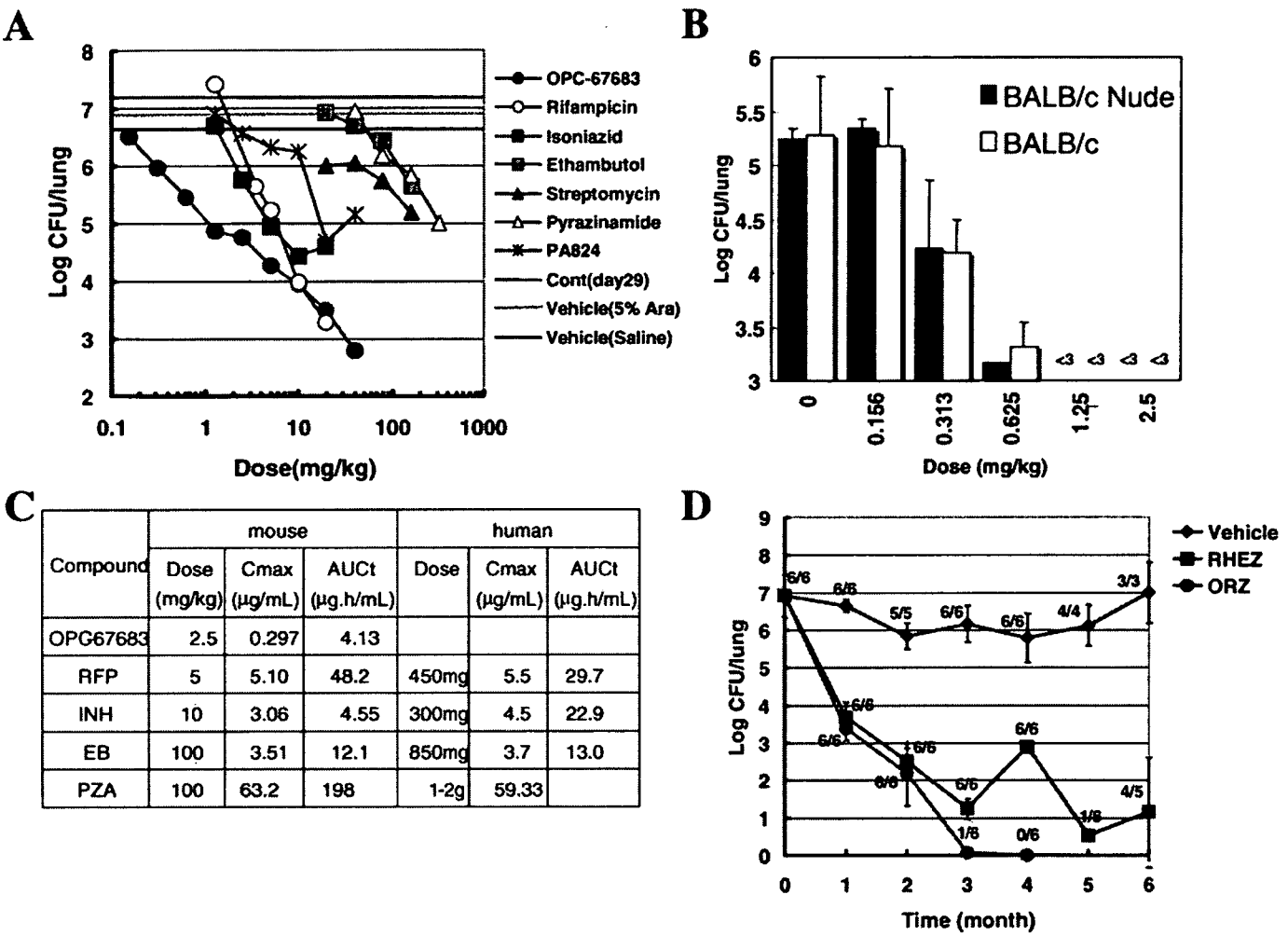
focused on compounds with activity against intracellular *M. tuberculosis*.

Mycobacteria are well known to be wax-rich bacteria, and a main component of the wax is mycolic acid, which is detected only in mycobacteria and not in gram-positive or gram-negative bacteria or in mammalian cells. Genome research of tubercle bacilli has verified this lipid richness, showing there to be almost 250 distinct enzymes involved in the lipid metabolism of tubercle bacilli [30]. In view of the important role of mycolic acid in mycobacteria, we searched for a compound that could inhibit mycolic acid synthesis and demonstrate potent anti-TB activity in vitro. We found OPC-67683 to have both inhibitory activity on mycolic acid biosynthesis and potent in vitro activity against *M. tuberculosis*, as indicated by its low MIC range across many strains, including MDR-TB. The IC_{50} values of OPC-67683 for mycolic acid subclasses were lower than those of INH, and these results correlated well with the in vitro anti-tubercular activity of OPC-67683 and INH. The anti-tubercular activity of nitro-imidazooxazole derivatives correlated well with their inhibitory activity against mycolic acid biosynthesis [11]. We therefore concluded that the inhibitory activity of OPC-67683 against mycolic acid synthesis was a mechanism of action attributable to killing mycobacteria at least as potently as INH.

As *M. tuberculosis* can grow not only facultatively but also as intracellular organisms that survive and multiply in macrophages of the infected host, we consider it important that a compound is also able to kill intracellular TB and that such activity should correlate with a shortened treatment duration and could be an important factor in the treatment of LTBI. We therefore examined the killing activity against intracellular TB in macrophage-derived THP-1 cells. Among the tested compounds, OPC-67683 demonstrated the most potent killing activity. The killing activity of OPC-67683 at 0.1 μg/ml was similar to that of RFP at 3 μg/ml and was superior to that of INH and PA-824. The intracellular potency of antibiotics is commonly evaluated in vitro using continuous exposure rather than in animal models due to their often-rapid elimination, depending on the plasma half-life. OPC-67683 was able to demonstrate potent in vitro killing ability even at short exposure times. These results indicate that OPC-67683 would likely exert strong antibiotic activity against intracellular TB in patients even at short exposure times, which could be an advantage in intermittent treatment.

PA-824 has been reported to be a prodrug metabolized to its active form by mycobacterium [31]. Recently, Manjunatha et al reported that Rv3547 acts as the catalytic enzyme for PA-824, but the role of Rv3547 within mycobacterium is not yet clear [32]. Similarly, OPC-67683 also requires metabolic activation by *M. tuberculosis* in order for the anti-TB activity to be exerted. Experimentally isolated OPC-67683-resistant mycobacterium did not metabolize the compound. We confirmed a mutation in the Rv3547 gene among the resistant organisms, indicating Rv3547 to be a key enzyme involved in activating OPC-67683, as it was for PA-824 (unpublished data). According to Manjunatha et al, the metabolites of PA-824 have not yet been identified. With OPC-67683, however, the main metabolite produced in the presence of *M. tuberculosis* was identified as a non-active desnitro-imidazooxazole. This result suggests that Rv3547 possesses a reduction potency of the nitro residue and that an intermediate between OPC-67683 and the desnitro-imidazooxazole could be the active form. After mixing radioactive OPC-67683 with viable mycobacterium, nearly 20% of the radioactive substances were not recovered. In contrast, after treating OPC-67683-resistant mycobacterium, nearly 100% of radioactivity was recovered. The action mechanism of metronidazole derivatives against *H. pylori* has been reported to be due to the production of a radical intermediate [33]. This information suggests the possibility that a radical intermediate that appears as the intermediate for the metabolism of a nitro residue covalently binds to the target molecule. If this hypothesis is correct, it could well explain the strong post-antibiotic effect seen with OPC-67683 against intracellular mycobacterium, a property considered necessary to kill latent TB.

The therapeutic efficacy of OPC-67683 was evaluated in vivo in an experimental chronic TB mouse model. In this



Compound	mouse			human		
	Dose (mg/kg)	Cmax (μg/mL)	AUCt (μg.h/mL)	Dose	Cmax (μg/mL)	AUCt (μg.h/mL)
OPG67683	2.5	0.297	4.13			
RFP	5	5.10	48.2	450mg	5.5	29.7
INH	10	3.06	4.55	300mg	4.5	22.9
EB	100	3.51	12.1	850mg	3.7	13.0
PZA	100	63.2	198	1-2g	59.33	

Figure 4. Effects of OPC-67683 in an Experimental Mouse Model of TB

(A) ICR mice were inoculated intravenously with *M. tuberculosis* Kurono. After 28 d, test compounds were administered orally once daily for 28 d (OPC-67683: 40–0.156 mg/kg, RFP: 20–1.25 mg/kg, INH: 20–1.25 mg/kg, EB: 160–20 mg/kg, SM: 160–20 mg/kg, PZA: 320–40 mg/kg, and PA-824: 40–1.25 mg/kg; 2-fold dilution). Mean value ($n = 5$) of $\log_{10}$ CFU was plotted.

(B) BALB/c standard and nude mice were inoculated intravenously with *M. tuberculosis* Kurono. From the following day, OPC-67683 was administered orally once daily for 10 d (OPC-67683: 10–0.313 mg/kg, 2-fold dilution). The bar was expressed as mean value and SD ($n = 5$) of $\log_{10}$ CFU.

(C) The doses of conventional drugs used for evaluating regimen are summarized in this table. The doses set up for using the plasma C_{max} achieved in mice TB model is equivalent to that achieved in humans at the clinical dose.

(D) ICR mice were inoculated intratracheally with *M. tuberculosis* Kurono. After 28 d, mice were treated for 2 mo with a combination of OPC-67683, RFP, and PZA (ORZ), or RFP, INH, EB, and PZA (RHEZ) (intensive treatment), and for an additional 2 mo with OPC-67683 and RFP or 4 mo with RFP and INH (maintenance treatment) (OPC-67683: 2.5 mg/kg, RFP: 5 mg/kg, INH: 10 mg/kg, EB: 100 mg/kg, and PZA: 100 mg/kg). Mean value and SD bar ($n = 6$) of $\log_{10}$ CFU was plotted. The fraction refers to the number of mice in which at least one colony was detected of the total number of surviving mice.

doi:10.1371/journal.pmed.0030466.g004

model, OPC-67683 exhibited the most potent anti-tubercular activity in comparison with the reference compounds. The viable bacterial counts in the lung were markedly reduced dose-dependently by OPC-67683 at 0.313 mg/kg and higher. A 95% reduction in bacterial load was achieved at 0.625 mg/kg. Furthermore, the efficacy of OPC-67683 in a TB model established using immunodeficient mice was similar to that seen using standard mice.

Treatment of TB requires combination therapy not only to shorten the treatment duration but also to prevent the development of resistance. The effects of OPC-67683 in combination with currently used TB drugs were therefore evaluated both in vitro and in vivo. OPC-67683 did not exert antagonistic effects in any of the tested combinations, and produced partial synergistic or synergistic effects when combined with RFP or EB in vitro. A combination regimen containing OPC-67683, RFP, and PZA produced a steady rapid reduction in bacterial load over the first 3 mo. These results suggest that a new regimen containing OPC-67683 could possibly be effective in shortening the clinical treatment duration.

Multiple-drug therapy is a common clinical practice, particularly in patients with concomitant diseases or conditions. However, whenever two or more drugs are administered concurrently, the possibility of drug interactions exists. Many drug interactions are clinically caused by inhibition of drug-metabolizing enzymes, such as CYPs, leading to decreased metabolic clearance and increased exposure to the inhibited drug [34–36]. Rifamycin derivatives such as RFP usually induce CYP3A4 enzymes, remarkably reducing the

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.
doi:10.1371/journal.pmed.0030466.t008

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).

Acknowledgments

We thank F. Tabusa and T. Sumida for their helpful discussions and expertise in their respective fields of medicinal chemistry and screening toxicology, and H. Ishikawa for his unrelenting support in bringing this TB drug project to a reality. We also acknowledge the many researchers, particularly M. Teshima, K. Ohguro, T. Hasegawa, and Y. Haraguchi, T. Koga, and several other staff members of Otsuka Pharmaceutical, the sponsoring company, for their many hours of dedication that have helped move OPC-67683 to its current stage. We also thank V. Lawlor for his editorial support.

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

Hirofumi Sasaki,† Yoshikazu Haraguchi,† Motohiro Itotani,† Hideaki Kuroda,† Hiroyuki Hashizume,‡ Tatsuo Tomishige,‡ Masanori Kawasaki,‡ Makoto Matsumoto,‡ Makoto Komatsu,† and Hidetsugu Tsubouchi*,†

Medicinal Chemistry Research Institute, Otsuka Pharmaceutical Co., Ltd., 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan, and Microbiological Research Institute, Otsuka Pharmaceutical Co., Ltd., 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan

Received August 8, 2006

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]

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.

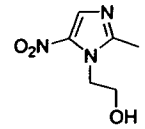
Chemistry

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

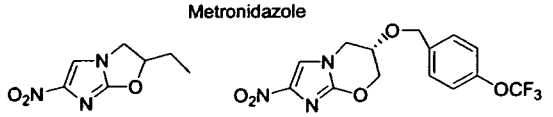
* To whom correspondence should be addressed. Phone: +81-88-665-2126. Fax: +81-88-665-6031. E-mail: h_tsubouchi@research.otsuka.co.jp.
† Medicinal Chemistry Research Institute.
‡ Microbiological Research Institute.

[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.

10.1021/jm060957y CCC: $33.50
Published on Web 12/06/2006



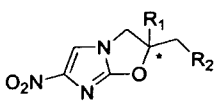
Metronidazole



1 **2**

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

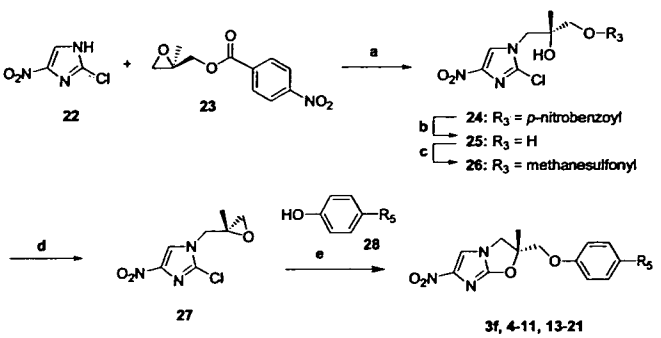
[a] MIC against *M. tuberculosis* $H_{37}Rv$. MIC of RFP = 0.1–0.39 μg/mL.

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*