Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis

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New therapeutic strategies are needed to combat the tuberculosis pandemic and the spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) forms of the disease, which remain a serious public health challenge worldwide1–2. The most urgent clinical need is to discover potent agents capable of reducing the duration of MDR and XDR tuberculosis therapy with a success rate comparable to that of current therapies for drug-susceptible tuberculosis. The last decade has seen the discovery of new agent classes for the management of tuberculosis3–5, several of which are currently in clinical trials6–8. However, given the high attrition rate of drug candidates during clinical development and the emergence of drug resistance, the discovery of additional clinical candidates is clearly needed. Here, we report on a promising class of imidazopyridine amide (IPA) compounds that block Mycobacterium tuberculosis growth by targeting the respiratory cytochrome bc1 complex. The optimized IPA compound Q203 inhibited the growth of MDR and XDR M. tuberculosis clinical isolates in culture broth medium in the low nanomolar range and was efficacious in a mouse model of tuberculosis at a dose less than 1 mg per kg body weight, which highlights the potency of this compound. In addition, Q203 displays pharmacokinetic and safety profiles compatible with once-daily dosing. Together, our data indicate that Q203 is a promising new clinical candidate for the treatment of tuberculosis.

To identify new antitubercular compounds, we screened various commercial chemical libraries using a phenotypic high-content screening technology in infected macrophages as previously described9. This technology has several advantages compared to traditional phenotypic screening approaches because it allows (i) screening under physiologically relevant conditions, which is notoriously challenging with the field10,11, (ii) selection of noncytotoxic compounds that effectively penetrate macrophages and (iii) selection of compounds that are poor substrates for macrophage-induced efflux mechanisms12. From a library of 121,156 compounds, we identified 106 active hits at a single concentration of 20 µM (70% inhibition cutoff). We confirmed that 96 compounds were active in dose-response studies, giving a hit rate of 0.08%. We selected for study a series of two IPA compounds, IPA01 and IPA02 (Supplementary Fig. 1) that were active against bacteria replicating inside macrophages and in culture broth medium in the low micromolar range (Supplementary Fig. 1). In addition, IPA01 was equally active against MDR clinical isolates (Supplementary Table 1), suggesting a mode of action distinct from rifampin and isoniazid, the two most active first-line antituberculosis drugs. Potential likeness to a drug, absence of cytotoxicity and equal activity against bacteria replicating inside macrophages (Supplementary Fig. 1) prompted us to initiate a lead optimization program. The synthesis and evaluation of 477 derivatives13 of the hit compound led to the optimized IPA Q203 (Fig. 1). Q203 was active against the reference strain M. tuberculosis H37Rv at a minimum
In *in vitro* and *ex vivo* activity of Q203. (a) Structure of Q203. (b) Activity of Q203 against *M. tuberculosis* replicating inside mouse macrophages RAW 264.7, expressed as percentage of infected macrophages and total number of macrophages remaining 5 d after infection. Data are expressed as the mean ± s.d. of triplicates for each concentration. (c) Activity of Q203 against GFP-labeled *M. tuberculosis* replicating in culture broth medium. Bedaquiline, isoniazid and moxifloxacin were used as positive controls. Data are expressed as the mean ± s.d. of triplicates for each concentration. RFU, relative fluorescence units. (d) Cytotoxicity of Q203 on human embryonic brain cells (SH-SY5Y cells) and human embryonic kidney cells (HEK293 cells), human embryonic liver cells (HepG2 cells) and human embryonic brain cells (SH-SY5Y cells). Data are expressed as the mean ± s.d. of triplicates for each concentration.

The metabolic stability of Q203 in microsomes and cryopreserved hepatocytes from human, monkey, rat and dog origin was high (Supplementary Table 5), suggesting that Q203 may achieve good blood exposure in humans. Because any new antitubercular drug will be given clinically in combination with other medications, the absence of drug-drug interactions is crucial. Q203 did not inhibit any of the cytochrome P450 (CYP450) isoenzymes tested, nor did it induce human pregnane X receptor (hPXR) activation (Supplementary Table 5). In addition, it was not a substrate or an inhibitor for the efflux transporter P-glycoprotein (Supplementary Table 5), indicating that it has low potential for drug-drug interaction.

Next, we determined the pharmacokinetic profile of Q203 in mice (Supplementary Table 6). Q203 had a bioavailability of 90% and a terminal half-life of 23.4 h. The volume of distribution was moderate (5.27 l per kg body weight), and the systemic clearance was low (4.03 ml min⁻¹ kg⁻¹). The drug concentration in lungs was two- to threefold higher than in the serum (Supplementary Table 7), which is a desirable property for an antitubercular drug. Given its desirable pharmacokinetic and safety profile, we assessed Q203 for *in vivo* efficacy. We initially evaluated Q203 in an acute mouse model of tuberculosis. After 4 weeks of treatment, we observed reductions of 90%, 99% and 99.9% in *M. tuberculosis* H37Rv bacterial load in the groups treated with Q203 at 0.4, 2 and 10 mg per kg body weight, respectively (Fig. 2b). Q203 was slow acting compared to isoniazid; the reduction in bacterial number was less than one order of magnitude in the first 2 weeks of treatment, but it was more than two orders of magnitude in the following 2 weeks. This profile might be explained by its pharmacokinetic properties or by its mode of action. Of note, bedaquiline displayed a similar time-dependent efficacy (Fig. 2b). We also observed that Q203 reduced the formation of lung granulomatous lesions (Fig. 2c–i). In untreated mice, the lung sections contained multiple tuberculous granulomatous foci (Fig. 2c), consisting predominantly of lymphocytes surrounding intra-alveolar macrophages (Fig. 2f). In isoniazid-treated groups, we observed a reduction in the size of the granulomatous foci; however, the number of the inflammatory lesions was comparable to that in the untreated control group (Fig. 2d,g,i). In contrast, we observed only a limited number of small granulomatous foci in the lungs of the mice treated with Q203 (Fig. 2c–i). Notably, other very effective drugs, such as bedaquiline, also have a strong beneficial effect on lung pathology.

To gain insight into the mode of action of Q203 and identify its molecular target, we selected spontaneous-resistant mutants to two different IPA derivatives, IPA04 and IPA05 (Supplementary Fig. 5).
Of 1 µM determined the spontaneous rate of mutation to IPA04 and IPA05 to be $2.4 \times 10^{-8}$, which indicates a low probability of emergence of resistant mutants. Spontaneous-resistant mutants selected directly on Q203 were also highly resistant to Q203 (Supplementary Fig. 6) and harbored a polymorphism T313A in qcrB, whereas we identified no mutation in qcrB in two pan-susceptible and three XDR clinical isolates (Supplementary Fig. 7). A similar polymorphism in qcrB was recently identified in Mycobacterium bovis bacillus Calmette-Guerin (BCG) mutants selected on a related IPA derivative14 that was less active than Q203 in inhibiting the growth of M. tuberculosis in vitro and not optimized for clinical use15. Notably, genetic approaches indicated that cytochrome $b_{c_1}$ is essential in M. tuberculosis19,20. The cytochrome $b_{c_1}$ complex is an essential component of the electron transport chain required for ATP synthesis. It catalyzes the electron transfer from ubiquinol to cytochrome $c$. This finding is in agreement with the previous observation that analogs of our hit compound could interfere with energy metabolism21,22. The cytochrome $b_{c_1}$ complex is the drug target of the antimalarial drug atovaquone23 and is known to be the target of nonselective inhibitors24. Although sequence similarities are relatively low, the overall structure of the cytochrome $b_{c_1}$ complex is highly conserved across species25–28. We therefore used the crystal structures of $b_{c_1}$ from the soil bacteria Paracoccus denitrificans25,
Rhodobacter sphaeroides26 and Saccharomyces cerevisiae27, as well as bovine bÌϵ (ref. 28), to infer the effect of Thr313 substitution. The cytochrome b subunit contains two ubiquinol reactive sites: the oxidation (Q9) site and the reduction (QÌá) site (Supplementary Fig. 8). The Thr313 residue was deduced to lie within the ubiquinol QÌá site; it aligns with the corresponding residues generally known as the ‘eÌ-’ region of the QÌá binding site24–27. Notably, several residues that have key roles in the binding of QÌá site inhibitors (for example, stigmatelin) or that are implicated in resistance to such inhibitors, or both, are located at the eÌ- region24–27 (Supplementary Fig. 8), suggesting a mode of action for Q203 similar to nonselective inhibitors acting at the QÌá site. Given the key role of cytochrome bÌϵ in the respiratory electron transport chain, we tested whether Q203 may interfere with ATP synthesis in M. tuberculosis. We found that Q203 triggered a rapid reduction in intracellular ATP at an IC50 of 1.1 nM (Fig. 3c). Under similar experimental conditions (see Online Methods), moffoxicin or streptomycin did not reduce the ATP pool size, whereas bedaquiline did (IC50 of 27.7 nM). Finally, Q203 was able to interfere with ATP homeostasis in hypoxic nonreplicating tuberculosis at an IC50 less than 10 nM (Fig. 3d). The rapid inhibition of ATP synthesis at low concentration strongly suggests that the inhibition of cytochrome bÌϵ activity is the primary mode of action of Q203.

Together, our data suggest that Q203 is a promising clinical candidate for the treatment of MDR and XDR tuberculosis, as it fits the desired target product profile for a new tuberculosis drug15. Its potency at low doses in the chronic mouse model of tuberculosis and its effect on energy metabolism suggest that Q203 may contribute to shortening the treatment time of tuberculosis29. In addition, our study further validates the proton-motive force and the process of ATP synthesis as targets for drug development against tuberculosis.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS


COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.
ONLINE METHODS

Synthesis of Q203 (6-chloro-2-ethyl-N-(4-(4-(4-(trifluoromethoxy)phenyl) piperidin-1-yl)benzyl) imidazo[1,2-a]pyridine-3-carboxamide). All reactions were carried out under an argon atmosphere in flame-dried glassware with magnetic stirring. Dimethylformamide was purified by passage through a bed of activated alumina. Reagents were purified before use. Purification of reaction products was carried out by flash chromatography using silica gel 60 (230–400 mesh, Merck). Analytical thin-layer chromatography was performed on 0.25-mm silica gel 60 F254 plates (Merck). Visualization was accomplished with ultraviolet light and iodine or potassium permanganate stain followed by heating. 1H-NMR spectra were recorded on a Varian 400 MHz spectrometer and are reported in p.p.m. using solvent as an internal standard (CDCl3 at 7.26 p.p.m.). Data are reported in the following format: (ap: apparent, s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet) or (b: broad). Coupling constants (J) in Hz, integration). Proton-decoupled 13C-NMR spectra were recorded on a Varian 100 MHz spectrometer and are reported in p.p.m. using solvent as an internal standard (CDCl3 at 77.2 p.p.m.). Liquid chromatography–mass spectrometry (LC-MS) data were obtained using a Waters 2695 LC and Micromass ZQ spectrometer. The purity of all biologically tested compounds was ≥ 95% by HPLC. Yields refer to purified products and are not optimized. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.74 g, 3.84 mmol), 1-hydroxybenzotriazole (0.21 g, 1.54 mmol), triethylamine (0.71 mL, 5.12 mmol) and 4-((4-(4-(trifluoromethoxy)phenyl)piperidin-1-yl)ethanamine (0.90 g, 2.56 mmol) were added to a solution of 6-chloro-2-ethylimidazo[1,2-a]pyridine-3-carboxylic acid (0.63 g, 2.83 mmol) in anhydrous DMF (10 mL) at room temperature and under stirring and then heated to 70 °C for 2 h under stirring. The reaction mixture was cooled to room temperature and evaporated. H2O (50 mL) was added to the crude residue, and the resulting solid was collected by filtration, washed with H2O (50 mL) and dried. The resulting crude compound was purified by flash column chromatography (n-hexane/ethyl acetate/methylenechloride, 1:1:1) and then recrystallized from EtOAc to give Q203 (0.98 g, 69%) as a white solid. 1H NMR (400 MHz, CDCl3) δ 8.13–1.97 (m, 2H), 2.84–2.87 (m, 2H), 2.93 (q, J = 7.6 Hz, 2H), 3.89–3.83 (m, 2H), 4.59 (d, J = 5.2 Hz, 2H), 6.01 (t, J = 5.2 Hz, 1H), 6.96–6.99 (m, 2H), 7.15 (d, J = 8.0 Hz, 2H), 7.24–7.30 (m, 5H), 7.52 (dd, J = 9.6, 0.8 Hz, 1H) and 9.53 (dd, J = 2.0, 0.8 Hz, 1H) p.p.m. 13C NMR (100 MHz, CDCl3) δ 161.2, 151.5, 151.4, 147.8, 144.8, 144.6, 128.9, 128.7, 128.3, 128.2, 126.3, 121.6, 121.2, 117.0, 115.4, 50.4, 43.3, 42.0, 33.4, 23.6 and 13.3 p.p.m. LC-MS with electrospray ionization: m/z 557 [M + H]+.

High-content screening assay in infected macrophages. The assay was performed as previously described39,40. Briefly, Raw 264.7 cells (American Type Culture Collection TIB-71) were infected with M. tuberculosis H37Rv-GFP at a multiplicity of infection of 2:1 and dispersed into 384-well plates. After 5 d of infection, macrophages were stained with Syto 60. Image acquisition was performed on an EVOscreen Mark III platform integrated with Opera. Bacterial load and macrophage number were quantified using proprietary image analysis software.9,30

Minimum inhibitory concentration determination. H37Rv-GFP was dispens ed into 384-well plates in 7H9 medium without glycerol. Mycobacterial growth was determined by measuring fluorescence intensity at 488 nm after 5 d of incubation. Alternatively, the MICs were determined using the resazurin susceptibility assay or a turbidity-based assay in 384-well plates. The assay was performed as previously described16. Briefly, mice were infected with a high dose of M. tuberculosis H37Rv. Dosing was initiated 6 d after infection. Drugs were administered orally for 3 d. Bacterial load in the lungs of infected mice was determined by colony-forming unit (CFU) enumeration. For the established mouse model, BALB/c mice were infected with 2 × 10^7 to 2 × 10^9 CFU of M. tuberculosis H37Rv by the intranasal route. Treatment was initiated 3 weeks after infection. Drugs were formulated in 20% TPGS and administered by oral gavage for 28 d, five times per week. Bacterial load in the lungs of infected mice was determined by CFU enumeration. For histopathology analysis, segments of the lungs were fixed with 10% neutral formalin, embedded in paraffin and processed for histology. Sections (5 μm) were stained with H&E. Histologic sections were used for morphologic analysis of the size and number of granulomas using an Image analyzer (Nikon, Japan). All animal studies were approved by the Institutional Animal Care and Use Committee of the Institut Pasteur-Korea in strict accordance with the Korean Animal Protection Law.

Selection of spontaneous-resistant mutants to IPA03, IPA04 and Q203. M. tuberculosis H37Rv was spread onto 7H11 agar plates containing 0.5 μM or 1 μM of either IPA03 or IPA04 in six distinct experiments. All colonies subjected to sequencing originated from different plates and represent independent biological events. Q203-resistant mutants were selected on 0.1 and 0.5 μM of Q203. The resistance phenotype to the compounds was confirmed by testing for a shift in MIC50 values.

Whole-genome sequencing. The whole-genome sequencing procedure was performed as previously described39. Whole-genome fragment libraries were prepared using the Paired-End Sample Preparation Kit (Illumina). Briefly, 5 μg of each genomic DNA sample was fragmented using the nebulization technique for 10 min with compressed air of 32 p.s.i. The ends were repaired with the addition of a base on the 3' end and by ligation of paired-end adaptor oligo mix. Samples were then gel-purified and selected for 200–base pair fragment size to be amplified by PCR, followed by gel purification. DNA samples were hybridized onto the flowcell using a Paired-End Cluster Generation Kit v1.0 and transferred to the Genome Analyzer Classic, and 48 cycles of sequencing were performed using the 36 Cycle Sequencing Kit v2. Data Collection Sequence Control software version 2.3 was used on the Genome Analyzer I, and Pipeline version 0.3.0 was used for analysis. The resulting 7.8–9.6 million reads were aligned to the M. tuberculosis H37Rv sequence using SOAP software. Nucleotide changes from the reference sequence were called in situations in which at least six separate reads called the alternate letter and in which the sum of the Solexa quality scores for the alternate letter was fivefold greater than the sum of the Solexa quality score for the reference letter. Insertions and deletions were verified by assembling de novo contigs with Velvet.

In vitro absorption distribution, metabolism, excretion and toxicity assays. Microsomal stability assay. Compounds (2 μM final in 0.2% DMSO) were incubated with 0.5 mg mL^-1 human (pool of 200, mixed gender, Xenotech), male dog, male rat or male mouse (BD Gentest) liver microsomes in potassium phosphate buffer. The reaction was initiated by the addition of NADPH and stopped either immediately or at 10, 20, 30 or 60 min for a precise estimate of clearance. A tripole quadrupole Quattro Premier mass spectrometer (Waters, Milford, MA) with electrospray ionization (ESI) was employed for sample analysis. Samples were passed through trapping cartridges (Acquity BEH RP18
CYP450 inhibition assays. The assay used individual fluorescent probe substrates with individual recombinant human cytochrome P450 (rhCYP) isozymes (BD Gentest) and a fluorescent detection according to previously published methods. The probe substrates (in 0.5% DMSO) used for each isozyme were as follows: 7-benzyloxy-4-(trifluoromethyl)-coumarin for CYP3A4, 3-[2-(N,N-diethyl-N-methylammonium)methyl]-7-methoxy-4-methylcoumarin (AMMC) for CYP2D6, 3-cyano-7-ethoxycoumarin (CEC) for 1A2 and 2C19 and 7-methoxy-4-(trifluoromethyl)-coumarin (MFC) for 2C9. Fluorescence was measured using Victor V multilabel plate reader. The IC_{50} was determined using an eight-point concentration curve with threefold serial dilution.

hERG patch clamp assay. CHO cells stably expressing hERG potassium channels (Aviva Biosciences) were used for the test. Compounds were tested at room temperature using the whole-cell patch-clamp techniques with a multiclamp 700B patch-clamp amplifier (Molecular Devices, US). Test compounds were tested in the dose-response curve from 0.3 to 30 μM, with each concentration tested in duplicate. Amitriptyline was used as positive control.

Mini-Ames assay. The mini-Ames assay was performed against Salmonella typhimurium strains TA98, TA100, TA1535 and TA1537 in the presence or absence of the hepatocyte S9 fraction (use to generate potential metabolites of the test compound). The bacterial strains were mixed in 2 ml soft agar supplemented with 0.5% NaCl, 0.5 mM L-histidine/biotin or 0.5 mM L-tryptophan, 80 μl of the test compound and 400 μl of the S9 fraction (for the activated assay) and poured on 5 mL of minimum glucose agar medium (1.5% agar and 2% glucose in Vogel-Bonner medium E) in six-well plates. Each treatment was done in triplicate, with the exception of untreated and negative controls, which were tested in sextuplicates. The plates were incubated at 37 °C for ~65 h. The number of revertant colonies was determined manually and recorded. The condition of the bacterial background lawn was evaluated for evidence of compound toxicity. Toxicity was scored relative to the negative control plates and was evaluated as a decrease in the number of revertant colonies per plate, a thinning or disappearance of the bacterial background lawn, or both. Potential compound precipitation was evaluated after the incubation period by visual examination and confirmed microscopically. Q203 did not show signs of precipitation up to 50 μM. 2-Aminoanthracene, 2-nitrofluorene, sodium azide, ICR-191 and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) were used as positive controls.

Micronucleus formation assay. The Chinese hamster ovary cell line CHO-WBL (originally obtained from Merck Research Laboratories, USA), with a modal chromosome number of 21, was employed in this assay. Every batch of cells was checked for stability of the modal chromosome number.

The cells were exposed to the test compound in the presence or absence of the S9 fraction (metabolic activation). Compounds were tested at various concentrations and in duplicate. After exposure to the compounds for 3 or 24 h, the cells were washed and incubated for an additional 24 h in the presence of 3 μM ml^{-1} of cytochalasin B. The cells were finally harvested on glass slides, stained with acridine orange and analyzed microscopically for the presence of micronuclei.

Mitomycin and cyclophosphamide were used as positive controls.

hPXR induction assay. The cell line DPX2, a derivative of HepG2 cells, harboring the human PXR gene and a luciferase-linked CYP3A promoter was used. The cell line was used in a panel of cell-based assays for a parallel assessment of CYP3A induction, metabolism and inhibition at the cellular level. To assess for hPXR induction, the Receptor Activation/Metabolism Assay Kit (puracel human pregnane X receptor (PXR) activation assay) was used according to the manufacturer’s recommendations. Rifampin, a known hPXR inducer, was used as a positive control. Data were expressed as fold induction compared to the level of induction obtained with rifampin.

Statistical analyses. All results are expressed as the mean ± s.d. Independent-sample Student’s t-tests were used to analyze differences between two groups. The significance level was set at P < 0.05.