www.sciencemag.org/cgi/content/full/1171583/DC1

Supporting Online Material for

Benzothiazinones Kill *Mycobacterium tuberculosis* by Blocking Arabinan Synthesis

Vadim Makarov, Giulia Manina, Katarina Mikusova, Ute Möllmann, Olga Ryabova, Brigitte Saint-Joanis, Neeraj Dhar, Maria Rosalia Pasca, Silvia Buroni, Anna Paola Lucarelli, Anna Milano, Edda De Rossi, Martina Belanova, Adela Bobovska, Petronela Dianiskova, Jana Kordulakova, Claudia Sala, Elizabeth Fullam, Patricia Schneider, John D. McKinney, Priscille Brodin, Thierry Christophe, Simon Waddell, Philip Butcher, Jakob Albrethsen, Ida Rosenkrands, Roland Brosch, Vrinda Nandi, Sowmya Bharath, Sheshagiri Gaonkar, Radha K Shandil, V. Balasubramanian, Tanjore Balganesh, Sandeep Tyagi, Jacques Grosset, Giovanna Riccardi, Stewart T. Cole*

*To whom correspondence should be addressed. E-mail: stewart.cole@epfl.ch

Published 19 March 2009 on *Science* Express
DOI: 10.1126/science.1171583

This PDF file includes:

Other Supporting Online Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/1171583/DC1)

Movies S1 and S2

Supplementary materials

Materials, Methods and Notes

Chemistry

Standard procedures were applied as described previously (*1, 2*). Purity of compounds was >96%.

Bacterial strains, growth conditions and plasmids. *M. tuberculosis* H37Rv was used as the reference strain. The recombinant strain of *M. tuberculosis* H37Rv expressing the green fluorescent protein (H37Rv-GFP) bears an integrative plasmid (based on Ms6) carrying a *gfp* gene constitutively expressed from the promoter *pBlaF*. Both strains were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80 and albumin-dextrose-catalase (ADC) or on solid Middlebrook 7H11 medium (Difco) supplemented with oleic acid-albumin-dextrose-catalase (OADC). When required, the media were supplemented with the following concentrations of antibiotics: 20 μg/ml of kanamycin, 50 μg/ml of hygromycin. To assess BTZ efficacy on non-growing cells, the streptomycin-dependent model (our unpublished work) and the nutrient starvation model (*3*) were used. Drug susceptibility to clinical isolates, including MDR- and XDR-strains, was performed at the Central Institute for Tuberculosis, Moscow and at the National Reference Center for Mycobacteria, Borstel.

MIC/MBC determination. Depending on the setting and requirement, MICs for antituberculosis drugs were determined using the BACTEC TB System (Becton-Dickinson Diagnostic Instrumentation Systems, Sparks, Md.), the micro-broth dilution method, the proportion method or the resazurin-reduction method (*4*). The MIC was defined as the lowest drug concentration that prevented growth of 99% of the cells and the MBC as the lowest concentration that prevented colony formation on solid medium. Generally the MIC and MBC for BTZ were near-identical.

Transcriptomics. *M. tuberculosis* H37Rv was grown to mid-log phase at 37˚C in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween 80 and albumin-dextrose-catalase (ADC), and exposed to 30 ng/ml BTZ043 for 4h alongside a carrier control. Mycobacterial RNA was extracted using the GTC/Trizol method as developed by Mangan *et al*. (*5*), and DNase-treated and purified using RNeasy columns (Qiagen). A *M. tuberculosis* whole genome microarray, generated by the Bacterial Microarray Group at St. George's (ArrayExpress accession number A-BUGS-23; http://bugs.sgul.ac.uk/A-BUGS-23), was utilized to profile the *M. tuberculosis* response to BTZ. Hybridizations were conducted as previously described (*6*), using *M. tuberculosis* genomic DNA as a common reference. Three biological replicates of BTZ-treated and control cultures were hybridized in duplicate. The hybridized slides were scanned sequentially at 532 nm and 635 nm corresponding to Cy3 and Cy5 excitation maxima using the Affymetrix 428 Array Scanner (MWG). Comparative spot intensities from the images were calculated using Imagene 5.5 (BioDiscovery), and imported into GeneSpring GX 7.2 (Agilent Technologies) for further analysis. The array data were normalized to the 50th

percentile of all genes detected to be present on the array, and filtered to include only genes flagged to be present on 80% of the arrays. Significantly differentially expressed genes were identified between control and BTZ-treated *M. tuberculosis* using t-test (p-value <0.05 with Benjamini and Hochberg multiple testing correction) and a fold change of >1.5. Hypergeometric probability (p-value <0.001) was used to identify similar drug exposure signatures derived from previously published datasets (6). Fully annotated microarray data has been deposited in BμG@Sbase (accession number: E-BUGS-80; http://bugs.sgul.ac.uk/E-BUGS-80) and also ArrayExpress (accession number: E-BUGS-80).

Proteomics. *M. tuberculosis* H37Rv was grown in triplicate cultures to OD_{580} 0.3-0.4, with or without 20 ng/ml of BTZ043, and harvested by centrifugation after 48 h of culturing. The bacterial pellet was washed twice in PBS, resuspended in 10 mM Tris, 250 mM sucrose buffer, pH 7.0, and broken with glass beads using a Mini-Beadbeater (Biospec products). The lysates were sterile filtered and protein concentration determined by the 2-D Quant kit (GE Healthcare). Sixty μg of each lysate was prepared for 2-D fluorescence difference gel electrophoresis (2-D DIGE) by the 2-D Clean-up kit (GE Healthcare) and dissolved in 30 mM TRIS, 7 M Urea, 2 M Thio-Urea, 4% CHAPS, pH 8.5. Cy2, Cy3 and Cy5 minimal labelling was performed according to the manufacturer's instructions (GE Healthcare), and 2-D electrophoresis was performed as previously described (*7*). Each 2-D gel included a control and a BTZ043-treated sample, as well as a Cy2-labeled internal standard (a pool of all samples in the experiment) to allow correction of inter-gel variation. After electrophoresis, gels were scanned on a Typhoon 9410 gel imager and spot images were analyzed with the Image Master Platinum 2.0 software (GE Healthcare). The Cy2-labeled standard was used for normalization by the default normalization algorithm, and spots, which displayed more than 2-fold intensity difference, were selected for identification. The 2-D DIGE gels were thereafter silver stained (*7*), and the relevant spots excised and identified by MALDI-TOF MS.

Comparative analysis of the proteome following BTZ-treatment revealed changes in the levels of several proteins. Consistent increases were seen with the INH-inducible protein IniB (Rv0341) and fumarate hydrolase (Rv1098c), while decreased abundance was observed for two conserved hypothetical proteins (Rv0831c, Rv3716c), and the exported proteins Apa (alanine and proline-rich protein, Rv1860), PepA (serine protease, Rv0125), MPT64 (Rv1980c), Antigen 85B (Rv1886c) and MPT51 (Rv3803c). Most of these changes mirrored those detected by transcriptomics and are hallmarks of cell wall damage.

Intracellular activity of BTZ. Raw 264.7 macrophages (ATCC # TIB-71) (1.5 x 10^8 cells) were infected with H37Rv-GFP at an MOI of 1:1 in 300 ml of RPMI 1640 (Gibco) with 10% heat-inactivated fetal calf serum (Gibco) for 2 h at 37 °C with shaking (100 rpm). After two washes by centrifugation at 1100 rpm (Beckman SX4250, 165 g) for 5 min, the remaining extracellular bacilli from the infected cells suspension were killed by a 1 h amikacin (20 μM, Sigma, A2324-5G) treatment. After a final centrifugation step, cells were dispensed with the Wellmate (Matrix) into 384-well Evotec plates (#781058) preplated with 10 μl of the respective

compound diluted in cell medium. For each compound, a series of 16 two-fold dilutions in quadruplicate was tested. Infected cells were then incubated in the presence of the compound for 5 days at 37 °C, 5% CO_2. Then, macrophages were stained with SYTO 60 (Invitrogen, S11342) followed by plate sealing and image acquisition. Confocal images were recorded on an automated fluorescent confocal microscope Opera™ (PerkinElmer) using a 20X-water objective (NA 0.70). Mycobacteria-GFP are detected using a 488-nm laser coupled with a 535/50 nm detection filter (green channel, GFP excitation) and cells labeled with Syto 60 are identified with 635-nm laser coupled with a 690/40 nm detection filter (red channel, Syto 60 excitation). Four independent fields are recorded for each plate well and each image was then processed using dedicated in-house image analysis software (IM developed by A. Genovesio) to determine five parameters (cell number, cell surface, infected cell number, green objects surface, green surface in infected cells corresponding to bacterial load). Briefly, the algorithm (developed by D. Fenistein) first segments the cells on the red channel using a sequence of processing steps described elsewhere (*8*). A similar classification applied on the green channel on pixels above a certain threshold is used to identify green objects. The infected cell number is then defined as those cells having at least a given number of pixels (usually 3) whose intensity in the green channel is above the threshold. The surface of green objects correlates with the number of mycobacteria and its ratio to the total cell number is referred as bacterial load.

Typical results of high content screening are shown in fig. S1 where it can be seen that after 5 days, macrophages treated with BTZ043 were abundant and displayed very few GFP-expressing bacilli compared to cells from the negative control (DMSO) or cells treated with the amino-derivative, BTZ045, at the same concentration.

BTZ efficacy in the chronic model. Animal efficacy was determined in a standard mouse infection model. BALB/c mice were infected with a low bacillary load (~200 CFU) of *M. tuberculosis* H37Rv via aerosol. Treatment started four-weeks post infection. Mice were dosed by gavage with 37.5, or 300 mg of BTZ, per kg body weight, in carboxymethyl cellulose formulation (0.25%), once daily, six times/week, for four weeks. Control and treated mice were sacrificed, lungs and spleens homogenized and dilutions plated for enumeration of viable bacilli.

Pharmacokinetics. When administered to mice by gavage the half-life in serum of BTZ043 was found to be >2h, Tmax >0.5h, Cmax = 2 μg/ml and the AUC (h*ug/ml) = 4.6.

***In vitro* assays of DPA production.** For these assays, the preparation of enzymatically active membrane, cell wall-enriched fractions and the radiolabeled substrate (p[^{14}C]Rpp) as well as the reaction mixtures, incubation conditions, and analysis of reaction products were essentially as described previously (*9*). In some experiments ~300 μg of protein from cell-free extracts of recombinant *E. coli* strains expressing Rv3790 and Rv3791 were used to catalyze the reaction. For this purpose the following *E. coli* strains were used as negative controls or sources of Rv3790 and

Rv3791: 1, C41(DE3)/pET28a; 2, C41(DE3)/pET28a-Rv3790-Rv3791; 3, BL21(DE3)/pET28a; 4, BL21(DE3)/pET28a-Rv3791; 5, BL21(DE3)/pET15b; 6, BL21(DE3)/pET15b-Rv3790, 7, BL21(DE3)pLysS/pET32b; 8, BL21(DE3)/pET32b-Rv3790 as indicated in the legend to Figure 3.

References and Notes to Supplementary Materials

1. V. Makarov, U. Möllmann, S. T. Cole, WO2007134625, (2007).
2. V. Makarov *et al.*, *J Antimicrobial Chemotherapy* **57**, 1134 (2006).
3. J. C. Betts, P. T. Lukey, L. C. Robb, R. A. McAdam, K. Duncan, *Mol Microbiol* **43**, 717 (2002).
4. J. C. Palomino *et al.*, *Antimicrob Agents Chemother* **46**, 2720 (2002).
5. J. A. Mangan, I. M. Monahan, P. D. Butcher, in *Functional Microbial Genomics,* B. W. Wren, N. Dorrell, Eds. (Academic Press, London, 2002), pp. 137-151.
6. G. R. Stewart *et al.*, *Microbiology* **148**, 3129 (2002).
7. I. Rosenkrands *et al.*, *Electrophoresis* **21**, 3740 (2000).
8. D. Fenistein, B. Lenseigne, T. Christophe, P. Brodin, A. Genovesio, *Cytometry A* **73**, 958 (2008).
9. K. Mikusova *et al.*, *J Bacteriol* **187**, 8020 (2005).
10. C. E. Barry, D. C. Crick, M. R. McNeil, *Infect Disord Drug Targets* **7**, 1 (2007).
11. B. A. Wolucka, *FEBS J* **275(11)**, 2691 (2008).

Supporting figures

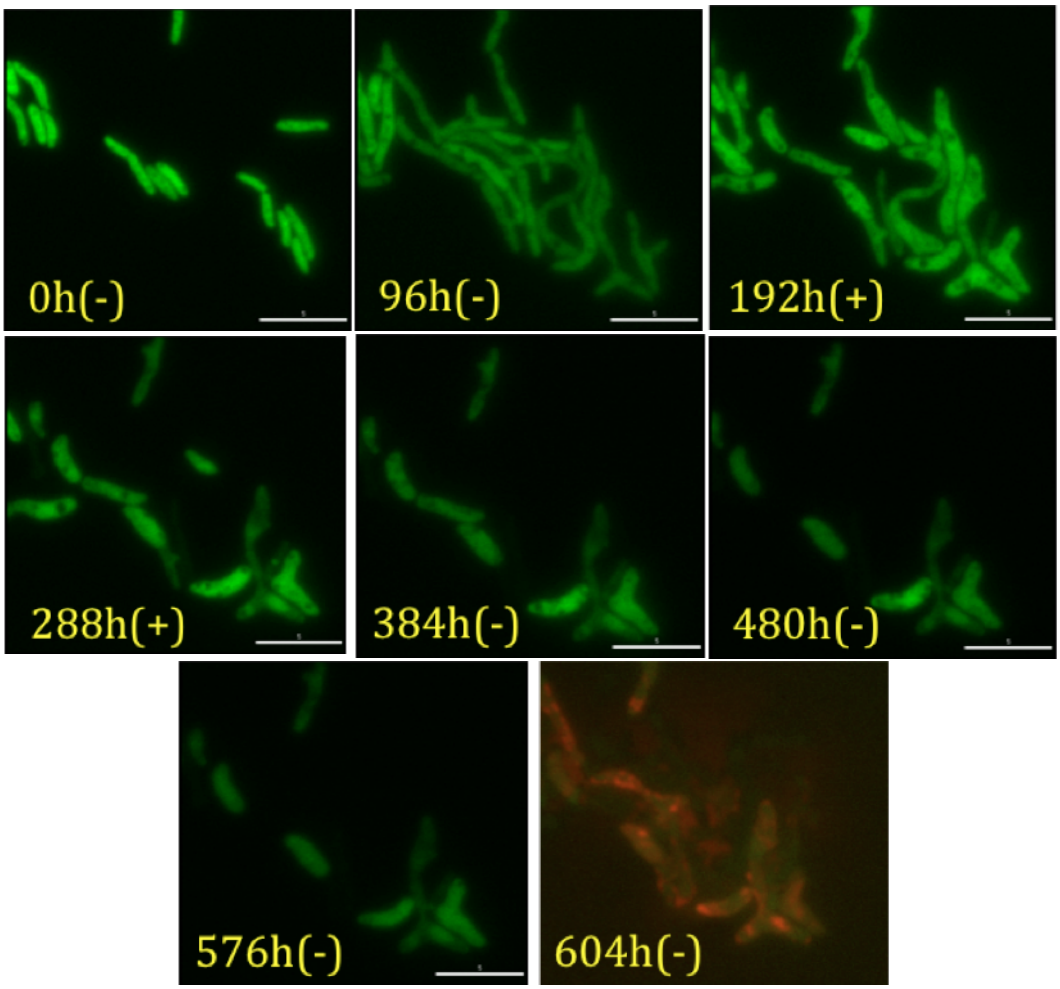


Fig. S1. Killing effect of BTZ043 on individual cells. *M. tuberculosis* growing in a microfluidics device was monitored at 2 h intervals by fluorescence microscopy (play movie S2). Representative frames are shown at 96 h intervals with (+) or without (–) drug. BTZ043 was added at 96 h (200 ng/ml) and maintained in the culture until 336 h when drug-free medium was introduced for the remainder of the experiment. Note the disappearance of GFP-labeled bacilli due to lysis within the first 96 h, the absence of regrowth and the extensive cellular damage as evidenced by propidium iodide uptake at the experiment's end (604 h). At this time all cells, including those positive for GFP, stained with propidium iodide. The white scale bar represents 5 μm.

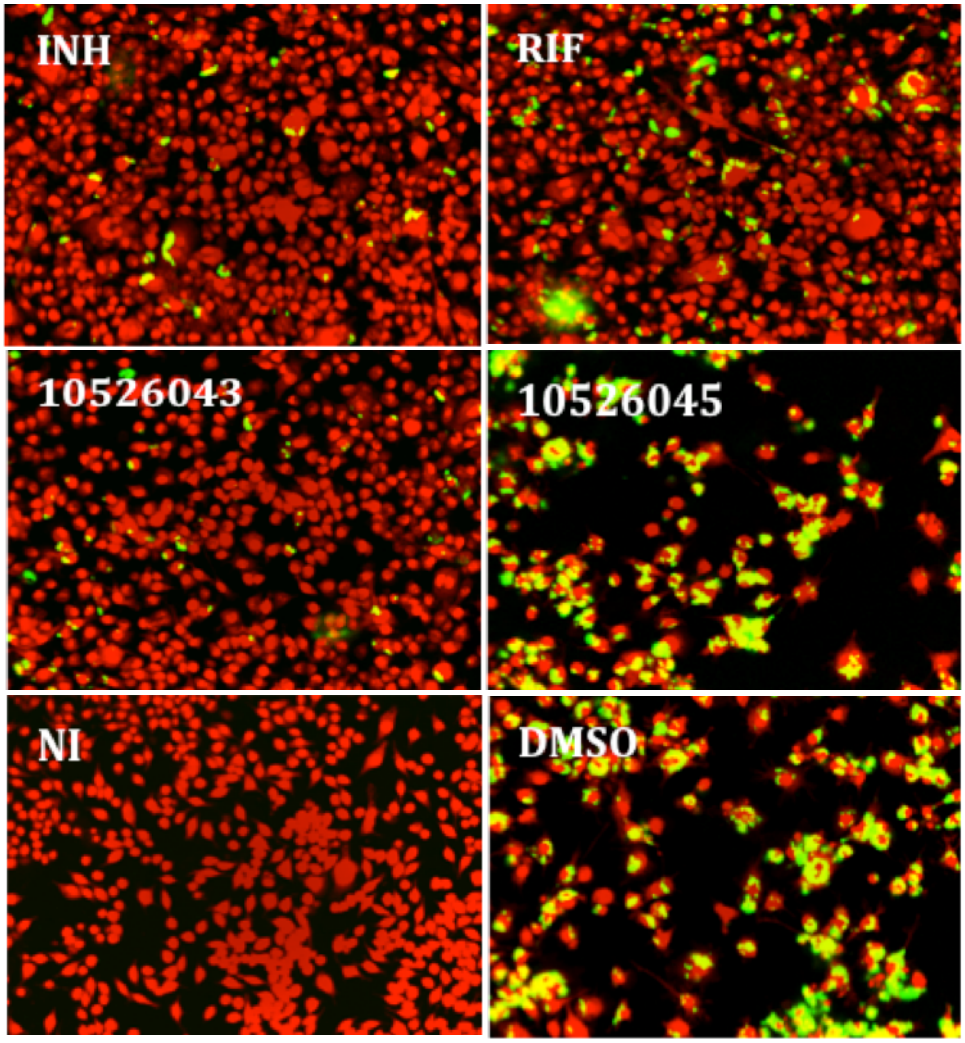


Fig. S2. Comparative BTZ efficacy in an *ex vivo* model. Serial dilution results from the *in vitro* growth fluorescence assay showing typical images of *M. tuberculosis*-infected Raw 264.7 cells incubated with BTZ compounds (10 ng/ml), INH, and rifampin (both 100 ng/ml) or the DMSO control. NI: Non-infected cells.

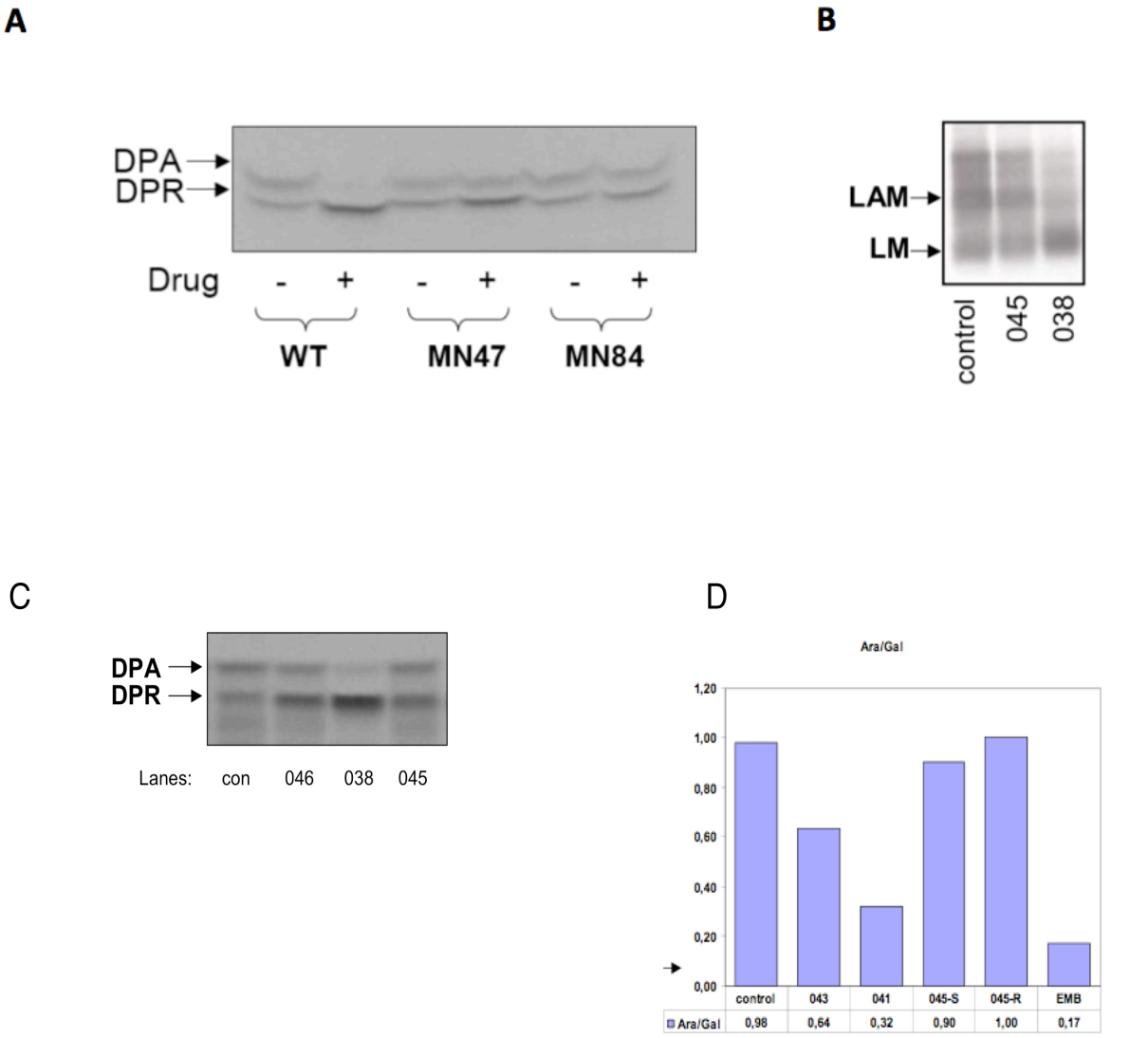


Fig. S3. Effect of BTZ on decaprenylphosphoryl arabinose (DPA) production *in vitro* and synthesis of arabinan polymers *in vivo*. **(A)** DPA production using membranes from BTZ-sensitive and resistant *M. smegmatis*. **(B)** Treatment with BTZ038 but not BTZ045 blocks lipoarabinomannan (LAM) production in growing cultures of *M. smegmatis*. LM, lipomannan. **(C)** Treatment with BTZ038 but not BTZ045 or BTZ046 blocks DPA production . **(D)** Treatment with BTZ043 or BTZ041, but not BTZ045S or R blocks arabinan production in *M. smegmatis* as shown by the Ara/Gal ratio. EMB was used as a positive control.

Figure S4

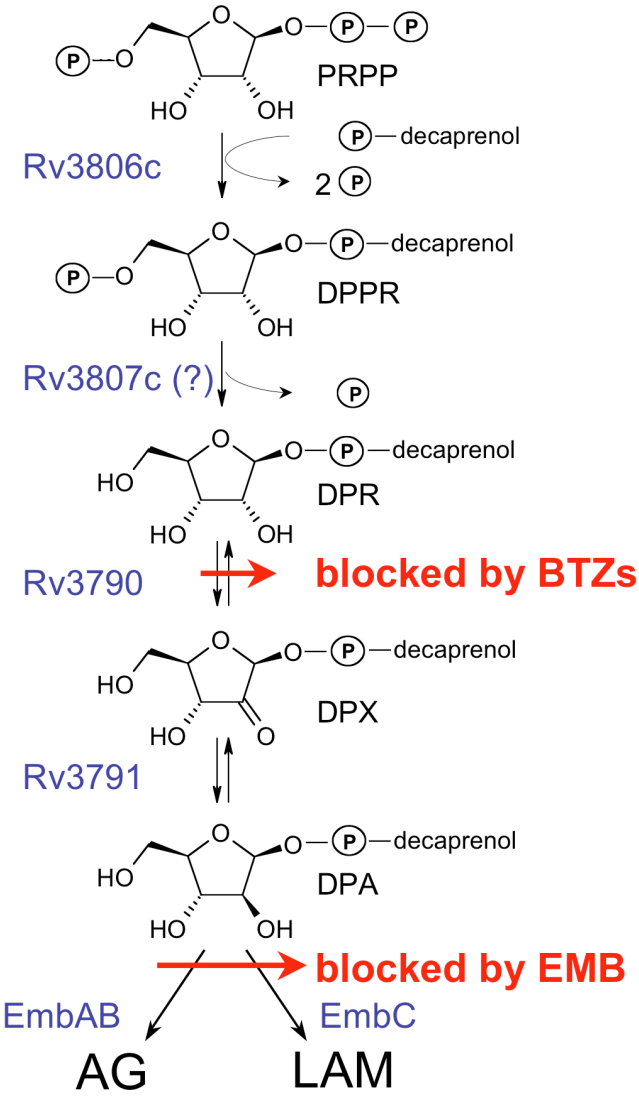


Fig. S4. Sites of action of BTZ and EMB. Proposed pathway, after (*10, 11*), of DPA production from DPR showing structures and sites of action of respective enzymes and drugs. DPA, decaprenylphosphoryl arabinose; DPR, decaprenylphosphoryl ribose; DPPR, decaprenylphosphoryl 5-phosphoribose; DPX, decaprenylphosphoryl "oxoderivative"; AG, arabinogalactan; LAM, lipoarabinomannan.

Movie S1

Time-lapse microscopy of BTZ-sensitive *M. smegmatis* strain (wild-type) exposed to BTZ043. Bacteria were cultivated at 37˚C in a continuous-flow microfluidic device and visualized using the 100X oil immersion objective. Images were acquired at 15 min intervals. Cells were fed standard 7H9 medium for 14.5 h and then exposed to BTZ043 at 0.1 μg/ml (25X MIC) for 74 h. This treatment resulted in lysis of the majority of bacteria. The compound was then washed out and the remaining cells were fed standard 7H9 medium for another 74 h (not shown here). The minority of cells that remained phase dense after BTZ exposure did not recover or resume growth after BTZ washout. When this experiment was performed with the BTZ-resistant *M. smegmatis* strain, MN84, these effects were not seen.

Movie S2

Time-lapse microscopy of BTZ-sensitive *M. tuberculosis* strain (H37Rv) exposed to BTZ043. Bacteria were cultivated at 37˚C in a continuous-flow microfluidic device and visualized using the 100X oil immersion objective. Images were acquired at 2 h intervals. Cells were fed standard 7H9 medium for 98 h and then exposed to BTZ043 at 0.2 μg/ml for 240 h. The compound was then washed out and the remaining cells were fed standard 7H9 medium for another 265 h. This treatment resulted in lysis of the majority of bacteria. The minority of cells that remained GFP-positive (a) and phase dense (b) after BTZ exposure did not resume growth after BTZ washout. At the end of the experiment the remaining bacteria were stained with propidium iodide (PI), which selectively stains cells with compromised cell wall integrity. The majority of the GFP-positive bacteria were also PI-positive, suggesting that the cell wall permeability barrier had been ruptured and these cells were most likely dead.

Supporting tables

Table S1. Main benzothiazinones, structures and MIC

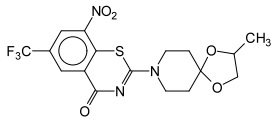
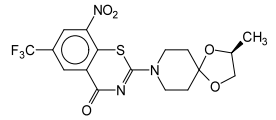
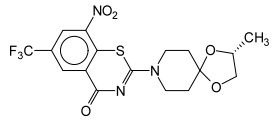
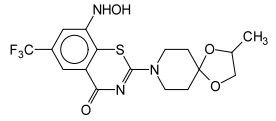
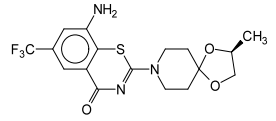
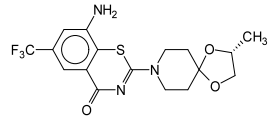
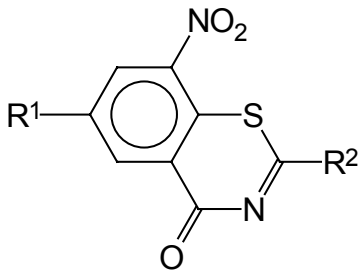
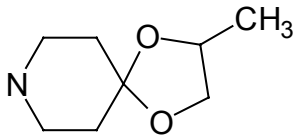
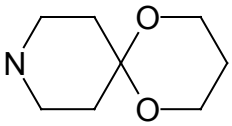
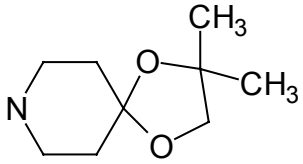
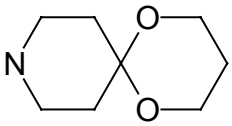
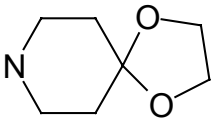
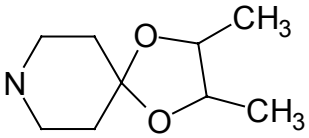
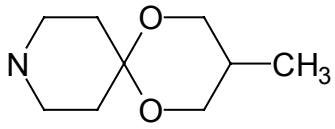
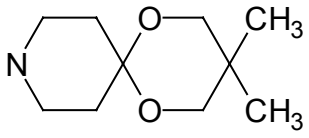
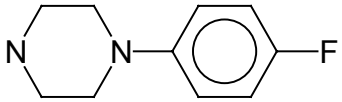
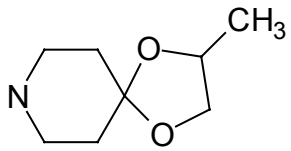
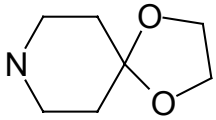
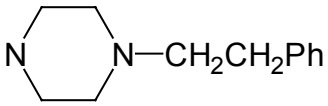
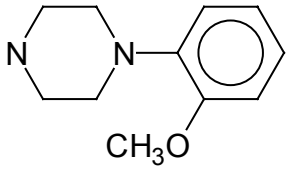
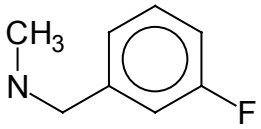
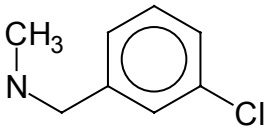
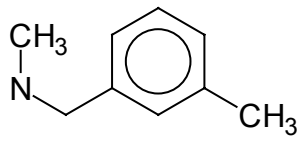
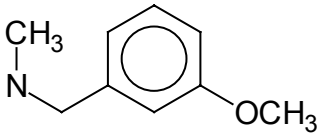
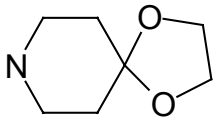
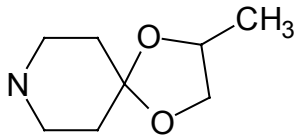
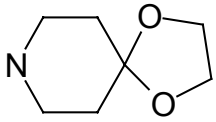
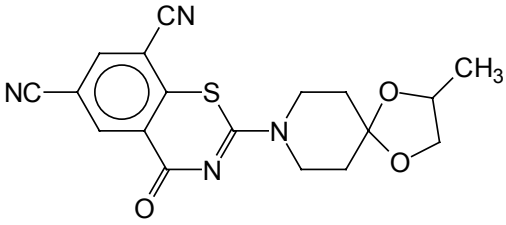
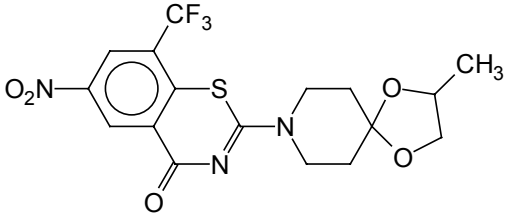
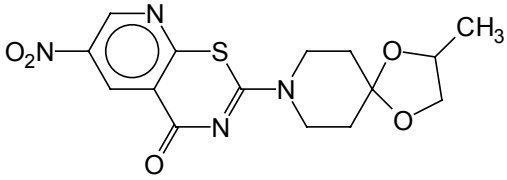
Compound	Name	Structure	*M. tuberculosis* MIC (ng/ml)	*M. smegmatis* MIC (ng/ml)
BTZ038 10526038	2-(2-methyl-1,4-dioxa-8-azaspiro[4.5]dec-8-yl)-8-nitro-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one		1	4
BTZ043 10526043	2-[(2S)-2-methyl-1,4-dioxa-8-azaspiro[4.5]dec-8-yl]-8-nitro-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one		1	4
BTZ044 10526044	2-[(2R)-2-methyl-1,4-dioxa-8-azaspiro[4.5]dec-8-yl]-8-nitro-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one		1	4
BTZ046 10526046	8-hydroxyamino-2-(2-methyl-1,4-dioxa-8 azaspiro[4.5]dec-8-yl)-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one		500	1,000
BTZ045S 10526045S	8-amino-2-[(2S)-2-methyl-1,4-dioxa-8-azaspiro[4.5]dec-8-yl]-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one		500	500
BTZ045R 10526045R	8-amino-2-[(2R)-2-methyl-1,4-dioxa-8-azaspiro[4.5]dec-8-yl]-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one		5,000	32,000

Table S2. SAR studies and MIC (ng/ml) for different BTZ against *M. tuberculosis* H37Rv and *M. smegmatis* mc^2155 by the resazurin reduction method



Compound	R^1	R^2	*M. tuberculosis*	*M. smegmatis*
10526032	CN		50	4
10526035	CN		50	16
10826050	CF_3		1.6	7.5
10526035	CN		50	16
10526040	CF_3		6	4
10626003	CF_3		50	2

10626033	CF_3		50	6
10626040	CF_3		25	6
10626045	CF_3		50	6
10626055	Cl		1	4
10726029	Cl		25	12.5
10626059	CF_3	$N(Et)CH_2Ph$	2	8
10726040	CF_3		5	<20
10726047	CF_3		5	<20
10726051	CF_3		2.5	<20
10726053	CF_3		1.2	<20

10726054	CF_3		1.2	<20
10726055	CF_3		>10	<20
10426177	NO_2		12.5	1
10426179	NO_2		12.5	1
10926007	H		>1000	>1000
10626011			>1000	>1000
10726070			n.t.	>1000
10726042			n.t.	>1000

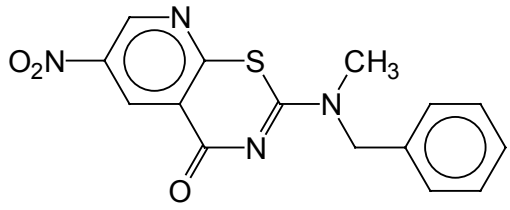
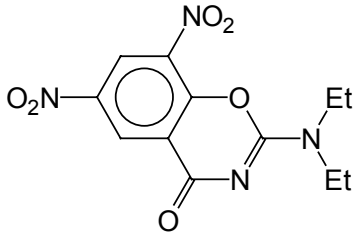
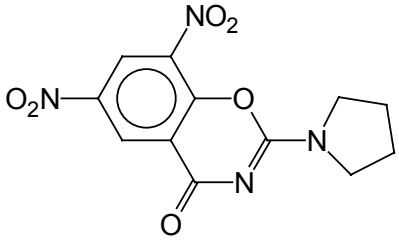
10726043		n.t.	>1000
10726008		>500	>1000
10726009		>500	>1000

Table S3. Microorganisms tested for BTZ susceptibility

Microorganism	Susceptibility
Bacillus subtilis	*R*
Candida albicans	*R*
Corynebacterium glutamicum	*S*
Corynebacterium pseudodiphtheriae	*S*
Corynebacterium pseudotuberculosis	*S*
Enterococococcus faecalis	*R*
Escherichia coli	*R*
Glomerella cingulata	*R*
Listeria monocytogenes	*R*
Mycobacterium aurum	*R*
Mycobacterium avium	*R*
Mycobacterium fortuitum	*S*
Mycobacterium marinum	*S*
Mycobacterium tuberculosis	*S*
Mycobacterium smegmatis	*S*
Mycobacterium vaccae	*S*
Nocardia farcinica	*S*
Proteus vulgaris	*R*
Pseudomonas aeruginosa	*R*
Pseudomonas putida	*R*
Rhodococcus spp.	*S*
Rhodococcus equi	*S*
Sporobolomyces salmonicolor	*R*
Staphylococcus aureus	*R*
***M. tuberculosis* complex**	
M. bovis BCG Pasteur	*S*
M. tuberculosis pan-sensitive clinical isolates (n>20)	*S*
M. tuberculosis mono-resistant clinical isolates (n>10)	*S*
M. tuberculosis MDR-strain 5055 (HSRE)	*S*
M. tuberculosis MDR-strain 6412 (HSRE)	*S*
M. tuberculosis XDR-strain 5253 (HSREKmRbZ)	*S*
M. tuberculosis XDR-strain 6418 (HSREKmO)	*S*

R, resistant; *S*, susceptible

Table S4. 60 genes significantly induced by 4h exposure to 30 ng/ml (x10 MIC) BTZ043.

Significantly differentially expressed genes were identified between carrier control and BTZ-treated *M. tuberculosis* using t-test (p-value <0.05 with Benjamini and Hochberg multiple testing correction) and a fold change of >1.5. Table ordered by chromosome position. Fully annotated methods and microarray data have been deposited in BμG@Sbase (http://bugs.sgul.ac.uk/E-BUGS-80) and ArrayExpress (E-BUGS-80).

Systematic	Gene Name	Fold Induction	t-test p-value	Putative Product
Rv0040c	*mtc28*	1.53	0.005347034	Secreted proline rich protein (28 kDa antigen); unknown function
Rv0129c	*fbpC*	2.08	2.41E-08	Secreted antigen 85 complex C; mycolyl transferase (fibronectin-binding protein); required for the biogenesis of trehalose dimycolate
Rv0179c	*lprO*	1.56	1.89E-04	Possible lipoprotein
Rv0230c	*php*	1.84	0.009346854	Probable phosphotriesterase (parathion hydrolase)
Rv0251c	*hsp*	1.60	0.001867878	Heat shock protein
Rv0278c	*PE_PGRS3*	1.49	0.01233815	PE_PGRS family protein; unknown function
Rv0341	*iniB*	2.07	0.003020786	Isoniazid inducible gene protein
Rv0366c	*Rv0366c*	1.87	0.016660627	Conserved hypothetical protein
Rv0789c	*Rv0789c*	1.65	4.74E-05	Hypothetical protein
Rv0848	*cysK2*	1.60	3.19E-05	Possible cysteine synthase; thought to be involved in cysteine biosynthesis
Rv0943c	*Rv0943c*	1.52	0.003830566	Probable monooxygenase; likely involved in cellular metabolism.
Rv0997	*Rv0997*	1.50	0.002822752	Hypothetical protein
Rv1041c	*Rv1041c*	1.55	0.003369446	Probable transposase; required for the transposition of an insertion element
Rv1057	*Rv1057*	1.65	0.004851358	Conserved hypothetical protein
Rv1075c	*Rv1075c*	2.10	9.01E-04	Conserved exported protein
Rv1076	*lipU*	2.31	0.004011884	Possible lipase; involved in lipid hydrolysis
Rv1157c	*Rv1157c*	1.76	3.65E-06	Conserved hypothetical Ala/Pro-rich protein
Rv1158c	*Rv1158c*	1.79	2.23E-04	Conserved hypothetical Ala/Pro-rich protein
Rv1217c	*Rv1217c*	1.56	1.25E-04	Probable tetronasin-transport integral membrane protein; involved in active transport of tetronasin across the membrane (export)
Rv1218c	*Rv1218c*	1.49	0.005966295	Probable tetronasin-transport ATP-binding protein; involved in active transport of tetronasin across the membrane (export)
Rv1221	*sigE*	1.70	0.007598866	Alternative RNA polymerase sigma factor
Rv1251c	*Rv1251c*	1.51	0.003761201	Conserved hypothetical protein
Rv1433	*Rv1433*	1.62	2.39E-04	Conserved exported protein
Rv1497	*lipL*	1.68	7.37E-07	Probable esterase; involved in lipid metabolism
Rv1522c	*mmpL12*	1.51	0.016785787	Conserved transmembrane transport protein; involved in fatty acid transport
Rv1528c	*papA4*	2.26	0.012812947	Conserved polyketide synthase associated protein; involved in lipid metabolism
Rv1592c	*Rv1592c*	1.61	0.002755387	Conserved hypothetical protein
Rv1697	*Rv1697*	1.54	6.79E-07	Conserved hypothetical protein
Rv1812c	*Rv1812c*	1.55	0.004930035	Probable dehydrogenase; involved in cellular metabolism

Rv1987	*Rv1987*	1.73	5.11E-05	Possible chitinase; involved in the hydrolysis of chitin
Rv2052c	*Rv2052c*	1.85	8.21E-04	Conserved hypothetical protein
Rv2154c	*ftsW*	1.58	7.10E-04	FTSW-like protein
Rv2163c	*pbpB*	1.52	0.00161857	Penicillin-binding membrane protein; involved in peptidoglycan biosynthesis
Rv2165c	*Rv2165c*	1.64	0.00285096	Conserved hypothetical protein
Rv2166c	*Rv2166c*	1.59	1.20E-04	Conserved hypothetical protein
Rv2320c	*rocE*	1.51	0.007492314	Cationic amino acid transport integral membrane protein; involved in transport of cationic amino acid (especially arginine and ornithine) across the membrane
Rv2410c	*Rv2410c*	1.56	7.64E-04	Conserved hypothetical protein
Rv2522c	*Rv2522c*	1.72	0.012727789	Conserved hypothetical protein
Rv2525c	*Rv2525c*	1.60	1.17E-04	Conserved hypothetical protein
Rv2550c	*Rv2550c*	1.81	0.007165412	Hypothetical protein
Rv2721c	*Rv2721c*	2.02	0.003378887	Conserved transmembrane; alanine and glycine rich protein
Rv2748c	*ftsK*	1.56	0.003204057	Cell division membrane protein; involved in cell division process
Rv2846c	*efpA*	1.59	0.0130867	Integral membrane efflux protein; involved in transport of undeterminated substrate (possibly drug) across the membrane (export)
Rv2894c	*xerC*	1.69	1.72E-04	Integrase/recombinase; participates in the site-specific recombination
Rv2927c	*Rv2927c*	1.61	2.44E-06	Conserved hypothetical protein
Rv3096	*Rv3096*	1.65	0.001342384	Conserved hypothetical protein
Rv3209	*Rv3209*	2.20	6.38E-04	Conserved hypothetical threonin and proline rich protein
Rv3258c	*Rv3258c*	1.50	0.010010552	Conserved hypothetical protein
Rv3310	*Rv3310*	1.78	1.65E-06	Possible acid phosphatase; involved in cellular metabolism
Rv3395A	*Rv3395A*	1.52	0.002287227	Probable membrane protein
Rv3405c	*Rv3405c*	1.55	0.016951786	Possible transcriptional regulatory protein
Rv3413c	*Rv3413c*	1.56	0.022156497	Hypothetical alanine and proline rich protein
Rv3435c	*Rv3435c*	1.64	0.005592665	Conserved transmembrane protein
Rv3530c	*Rv3530c*	1.60	0.01464131	Possible oxidoreductase; involved in cellular metabolism
Rv3675	*Rv3675*	1.81	0.00808929	Possible membrane protein
Rv3717	*Rv3717*	1.98	6.49E-07	Conserved hypothetical protein
Rv3756c	*proZ*	1.82	0.002801984	Possible osmoprotectant transport membrane protein; involved in active transport of glycine betaine/carnitine/choline/proline across the membrane (import)
Rv3810	*pirG*	1.60	0.002700412	Exported repetitive protein precursor; surface-exposed protein required for multiplication and intracellular growth
Rv3837c	*Rv3837c*	3.37	4.81E-05	Probable phosphoglycerate mutase; involved in cellular metabolism
Rv3918c	*parA*	2.00	1.17E-04	Chromosome partitioning protein; localises to both poles of the pre-divisional cell following completion of DNA replication

Table S5. *In vitro* ADMET (Adsorption, Distribution, Metabolism, Excretion, Toxicity) with selected BTZ.

Solution properties

Compound	Solubility (μM)
BTZ038	7.8
BTZ045	10.1

Partition Coefficient (log *P*)

Compound	log *P*
BTZ038	2.84
BTZ045	3.06

Plasma protein binding

Compound	Protein bound (%)
BTZ038	7.5
BTZ045	84.8

Mutagenicity - SOS chromotest

Compound	Mutagenicity
BTZ038	-
BTZ045	-

Mutagenicity - Ames test

Compound	Mutagenicity
BTZ038	-
BTZ045	-

Metabolic Stability (human liver microsomes)

Compound	Remaining (%)
BTZ038	77
BTZ045	79

Cytochrome P450 inhibition

Compound	Inhibition (%)				
	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A4
BTZ038	1	15	17	-1	17
BTZ045	8	-139	65	-1	31

QT prolongation K^+ channel (hERG)

BTZ038	low
BTZ045	low

Table S6. ***In vivo*** **toxicology of 10526043**
Summary of findings of chronic toxicity study (25 and 250 mg/kg doses, 30 days treatment) in BALB/c mice.

- No influence on dynamics of weight gain.
- Normal content of urea and creatinine in serum.
- Normal levels of protein, alanine aminotransferase and alkaline phosphatase.
- Slight reduction in aspartate aminotransferase level in mice receiving 250 mg/kg only.
- No effect on peripheral blood cell count.
- Macroscopic examination of internal organs revealed no difference to control.
- Morphological analysis of internal organs revealed no difference to control.
- Pathological analysis of internal organs revealed no difference to control except in liver of some animals at the dose of 250 mg/kg.
- Histological analysis of internal organs revealed no difference to control except in liver where at the dose of 250 mg/kg only some microfoci and inflammatory infiltrates were seen.
- No influence on blood pressure or heart rate was seen at various doses.
- No statistically significant effect on cardiac electrical activity was seen.
- No arrhythmia was detected.
- At the level of the GI tract, normal levels of basal secretion were seen and no diarrhea was induced. Likewise, no irritating and ulcerogenic effects were observed.