

In Vitro Effect of DFC-2 on Mycolic Acid Biosynthesis in *Mycobacterium tuberculosis* ^S

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DFC-2, a methyl 5-[2-(dimethylamino)ethoxy]-7,12-dioxo-7,12-dihydrodinaphtho[1,2-b:2',3'-d]furan-6-carboxylate, is reported to have antitubercular effects against *Mycobacterium tuberculosis*. At concentrations ranging from 0.19 to 0.39 µg/ml, DFC-2 inhibited both drug-susceptible and -resistant strains of *M. tuberculosis*. Microarray analyses were employed to gain insights into the molecular mechanisms of DFC-2's action in *M. tuberculosis*. The most affected functional gene category was "lipid biosynthesis," which is involved in mycolic acid synthesis. The decrease in transcription of genes related to mycolic acid synthesis was confirmed by RT-PCR. Furthermore, we found that DFC-2 triggered a reduction in mycolic acid levels, showing a similar pattern to that of mycolic acid synthesis inhibitor isoniazid. These results may explain how this compound kills mycobacteria efficiently by inhibiting mycolic acid synthesis.

Keywords: DFC-2, mycolic acid, *M. tuberculosis*

Introduction

According to *Global Tuberculosis Report 2016* published by WHO, approximately 10.4 million people have contracted tuberculosis and 1.4 million of them have died as a result [1]. Today, treatment of tuberculosis is long and burdensome, requiring at least 6 months of multidrug chemotherapy [2]. The situation is further complicated by the emergence of multidrug-resistant (MDR) *M. tuberculosis* and extensively resistant (XDR) *M. tuberculosis*, and by the infection's fatal synergy with HIV/AIDS [2]. Thus, development of new antitubercular drugs is essential for effective treatment and overall cure of the disease.

We initiated a program to screen potential antitubercular compounds of natural origin that have a new structure and

mechanism capable of inhibiting the drug-resistant strains of *M. tuberculosis*. After extensive screening of 800 compounds of plant origin, we identified plumbagin (a naphthoquinone derivative) as a promising candidate, which was purified from *Plumbago auriculata*. Next, we had screened a library of compounds that are similar in structure to plumbagin and finally found an active compound, DFC ([5-(3-dimethylaminopropoxy)-7,12-dioxo-7,12-dihydrodinaphtho [1,2-b:2',3'-d] furan-6-carboxylic acid methyl ester)] [3]. To extend the research on DFC as a new class of antitubercular agent, we designed a combinatorial library based on the dinaphthofurandione structures of DFC. A series of DFC analogs were synthesized and tested for in vitro antitubercular activity and cytotoxicity. Among those analogs, methyl 5-[2-(dimethylamino)ethoxy]-7,12-dioxo-7,12-dihydrodinaphtho

[1,2-b:2',3'-d]furan-6-carboxylate (DFC-2) showed superior minimum inhibitory concentration (MIC) against drug-susceptible and -resistant *M. tuberculosis* [3]. The MIC of DFC-2 against the reference strain *M. tuberculosis* H37Rv in culture broth was 0.39 µg/ml, and it progressively killed *M. tuberculosis* inside macrophages at 1× MIC [3]. The 50 % cell cytotoxicity (CC₅₀) value of DFC-2 against L929 cells was 15.21 µg/ml, which gives this compound a selectivity index of 39 [3]. However, there has been no study that investigates the molecular mechanism of its action in *M. tuberculosis*. In this study, we have analyzed transcriptional profiles of *M. tuberculosis* in response to DFC-2 and evaluated its effects on mycolic acid biosynthesis.

Materials and Methods

Synthesis of DFC-2

DFC-2 was synthesized through a simple 3-step procedure (Fig. 1).

Synthesis of methyl-1,4-dihydroxy-2-naphthoate (1). To a stirred solution of 1,4-dihydroxy-2-naphthoic acid (25.0 g, 122.44 mmol) and NaHCO₃ (12.86 g, 153.05 mmol) in DMF (200 ml), CH₃I (26.07 g, 183.66 mmol) was added dropwise over a period of 10 min at 0°C. Then, the reaction mixture was stirred at RT for 16 h. The progress of the reaction was monitored by TLC. After completion, the reaction mixture was poured into ice-cold water (800 ml) and stirred for 10 min. The off-white solid formed was filtered, washed with water (300 ml), and dried under vacuum to give **1** (24.3 g, 91%) solid, mp 194–195°C; ¹H NMR (DMSO-d₆): δH 11.44 (s, 1H), 9.19 (s, 1H), 8.32 (d, J = 8.1 Hz, 1H), 8.18 (d, J =

8.1 Hz, 1H), 7.63–7.50 (m, 2H), 7.14 (s, 1H), 3.98 (s, 3H) ppm; ¹³C NMR (125 MHz, DMSO-d₆): δC 170.87, 153.04, 145.48, 129.37, 129.06, 126.75, 125.19, 123.54, 122.52, 105.08, 104.13, 52.90 ppm.

Synthesis of methyl-5-hydroxydinaphtho [1,2-b;7,12-dione-6-carboxylate (2). A mixture of 2,3-dichloro-1,4-naphthoquinone (20.0 g, 88.09 mmol), **1** (28.83 g, 132.14 mmol), and K₂CO₃ (133.92 g, 968.99 mmol) in pyridine (1,000 ml) was heated to 90°C and maintained overnight. K₂CO₃ was filtered through a bed of celite, and after washing the bed, the filtrate was concentrated to 1/4 volume and poured into icewater (1,000 ml). The precipitated solid was filtered and washed with H₂O (300 ml), dried under vacuum, and then recrystallized from chloroform to give **2** (20.9 g, 64%) solid, mp 249–251°C; ¹H NMR (300 MHz, DMSO-d₆): δH 10.45 (s, 1H), 8.41 (d, J = 8.1 Hz, 2H), 8.17–8.11 (m, 2H), 7.94–7.89 (m, 2H), 7.86–7.78 (m, 2H), 4.0 (s, 3H) ppm.

Synthesis of methyl-5-(2-dimethylaminoethoxy) dinaphtho[1,2-b;2',3'-d]furan-7,12-dione-6-carboxylate (DFC-2). To a solution of **2** (25.0 g, 67.14 mmol) and 2-dimethylaminoethyl chloride hydrochloride (29.01 g, 201.43 mmol) in DMF (300 ml), K₂CO₃ (185.59 g, 1.34 mol) was added and then stirred for 16 h at 50°C. The reaction mixture was poured into icewater (900 ml) and extracted into CH₂Cl₂ (3 × 500 ml). The combined organic layers were washed with water (2 × 250 ml), dried over anhydrous Na₂SO₄, and evaporated to give brown residue, which was further purified by column chromatography (0.5/9.5 = MeOH/CH₂Cl₂ as eluent) to afford DFC-2 (16.08 g, 54%) solid, mp 164–167°C; ¹H NMR (500 MHz, CDCl₃): δH 8.55 (d, J = 8.35 Hz, 1H), 8.37 (d, J = 8.0 Hz, 1H), 7.81–7.75 (m, 5H), 4.33 (t, J = 5.65 Hz, 2H), 4.20 (s, 3H), 2.89 (t, J = 5.65 Hz, 2H), 2.45 (s, 6H) ppm; ¹³C NMR (125 MHz, CDCl₃): 180.4, 174.6, 166.8, 153.2, 151.8, 150.2, 134.2, 133.9, 133.2, 132.5, 128.9, 128.6, 127.2, 126.9, 125.2, 124.2, 122.1, 121.7, 118.6, 115.9, 74.6, 59.2, 52.9, 45.9 ppm.

Commercial Drugs

Isoniazid (INH), rifampicin (RIF), streptomycin sulfate salt (STR), pyrazinicarboxamide (PZA), ethambutol dihydrochloride (EMB), and ethionamide (ETH) were purchased from Sigma-Aldrich.

Microorganisms

MDR (KMRC 00116-00050), XDR (KMRC 00203-00197), and single drug-resistant (SDR) strains of *M. tuberculosis*, including those resistant to INH (KMRC 00120-00137), RIF (KMRC 00121-00341), PZA (KMRC 00130-00064), and STR (KMRC 00122-00123), were obtained from the Korean Institute of Tuberculosis (Korea). The H37Rv (ATCC 27294) and H37Ra (ATCC 35835) strains of *M. tuberculosis* were obtained from the ATCC.

Determination of Antimycobacterial Susceptibility In Vitro

Microdilution technique. A microdilution technique (MDT) using the colorimetric indicator resazurin was used to assess antitubercular activities previously [4–6]. We used the resazurin assay to determine the antitubercular activities of the compounds against several strains of *M. tuberculosis*. Middlebrook 7H9 broth

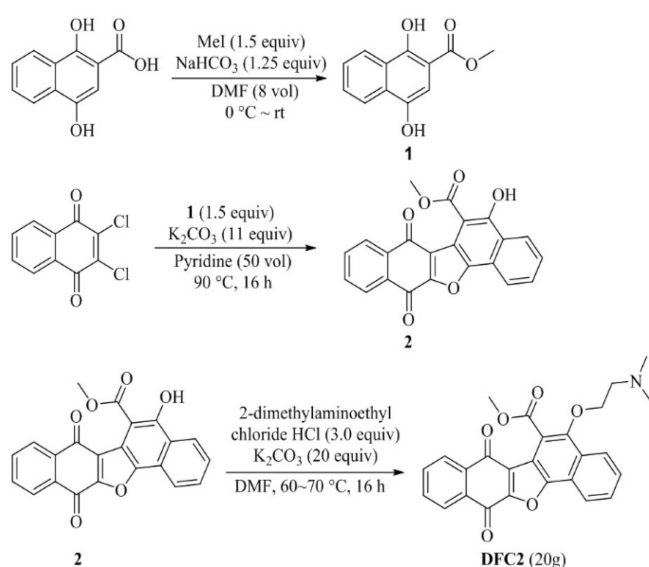


Fig. 1. Synthetic scheme of DFC-2.

(Difco) including 10% albumin-dextrose-catalase (ADC; BD) and 0.2% glycerol was used in the resazurin assay. In the test, 5 μ l of serial 2-fold drug dilutions were added into a 200 μ l inoculum (3×10^5 CFU/ml) at 12 concentrations, of 12.5 to 0.006 μ g/ml, in a 96-well plate. After 7 days of incubation, 20 μ l of 0.02% resazurin solution was added to each well, and color changes were assessed after a 48-h incubation at 37°C. A color change from blue to pink shows reduction of resazurin and thus shows bacterial growth. We defined the MIC_{MDT} for each respective strain as the lowest concentration of a compound that had no color change [5].

Tube dilution technique. A tube dilution technique (TDT) was previously used to evaluate antibacterial activities of various agents [7, 8]. We applied and standardized the TDT to assess the antitubercular activities of compounds against strains of *M. tuberculosis*. We determined the MIC_{TDT} of each strain of *M. tuberculosis* starting from bacterial culture at an OD_{600nm} = 0.3 after drug treatment for 7 days compared with drug free control groups.

Cell viability assay. Total ATP, an energy-dependent parameter, was used for rapid viability assessment after exposure of *M. tuberculosis* to antibiotics [9–11]. ATP analysis was assessed as previously described [12, 13]. Briefly, *M. tuberculosis* H37Rv was exposed to the test compounds from day 0 to day 5 at 37°C, and included with an equal volume of BacTiter-Glo Microbial Cell Viability Assay Kit reagent (Promega) and incubated in the dark for 10 min at 37°C. Luminescence was recorded on a multilabel plate reader (PerkinElmer).

RNA Isolation, RT-PCR, and Microarray

The drug concentrations and time points used were selected after considering previously published studies [14, 15]. After drug treatment, RNA samples from three biological replications were extracted with an RNAProtect bacteria reagent kit (Qiagen) as described by the manufacturer's protocol. RNA samples were reverse transcribed to cDNA with a cDNA synthesis kit (Bio-Rad) as described by the manufacturer's protocol. Primer pairs were designed for PCR confirmation of genes as described in Table 1. Copies of 16S rRNA were used to normalize the transcript levels of their respective genes [14, 16, 17]. Microarray assay was performed using 3 $\times$ 20K microarray chips (<http://Mycroarray.com>) from a commercial microarray service (Ebiogen, Inc., Korea).

Quantification of Mycolic Acids

To obtain cell wall mycolic acids from *M. tuberculosis*, previously reported protocols [16, 17] were slightly modified. Cell numbers were adjusted to OD_{600nm} = 0.3 and treated with concentrations of 0.01 $\times$ to 10 $\times$ MIC_{TDT} of DFC-2 and INH, respectively. After 5 days of incubation (37°C, 120 rpm), the cells were collected by centrifugation, followed by washing with PBS. The pellets were transferred to 2 ml of 25% methanolic potassium hydroxide (KOH) dissolved in methanol and water at 1:1) and mixed thoroughly for 30 sec. Then, all tubes were heated for 1 h at 90°C in an oven and then cooled to room temperature. To precipitate the mycolic acids,

2 ml of chloroform was added, followed by 1.5 ml of 6 N HCl, and then mixed thoroughly for 30 sec. The chloroform layer including the dissolved mycolic acids were then transferred by Pasteur pipette to new glass tubes and oven-heated at 80°C until dried. The resulting precipitate was dissolved in chloroform/methanol (2:1 (v/v)) and transferred into the autosampler for LC-MS/MS. For standardization and optimization of the MS/MS condition, mycolic acid from *M. tuberculosis* (Sigma Aldrich, Cat No. M4537) was used. Quantification of mycolic acids was evaluated by LC-MS/MS as previously described [15].

Electron Microscopy

Transmission electron microscopy (TEM). For TEM observations, the mycobacterial cells were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for overnight. After washing in 0.1 M phosphate buffer, the specimens were postfixed with 1% osmium tetroxide in the same buffer for 1 h. Then, the specimens were dehydrated with a graded series of ethyl alcohol and pure acetone. Specimens were embedded in Epon 812 and then polymerization was performed at 60°C for 3 days. Ultrathin sections (60–70 nm) were obtained by ultramicrotome (Leica Ultracut UCT, Germany). Ultrathin sections collected on grids (200 mesh) were examined under a transmission electron microscope (ZEISS LIBRA 120).

Scanning electron microscopy (SEM). For SEM observations, the mycobacterial cells were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for overnight. After washing in 0.1 M phosphate buffer, the specimens were postfixed with 1% osmium tetroxide in the same buffer for 1 h. The specimens were then dehydrated in ascending concentration of ethyl alcohol from 50% to 100% (12 h at each concentration). The specimens were then transferred to hexametyldisilazane and allowed to air dry. All samples were coated with gold using a sputter coater and examined on a LEO-1530 device (Carl Zeiss).

Results and Discussion

Confirmation of Antituberculosis Activity of DFC-2 In Vitro

To confirm the previous study that showed antitubercular activity of DFC-2 [3], we conducted a MDT, a colorimetric microtiter plate-based drug susceptibility assay [18]. The antitubercular activity of DFC-2 was compared with that of five first-line drugs: INH, RIF, STR, PZA, and EMB. Apparently, the MIC data were reproducible, with only minor variations (Fig. 2). The MIC_{MDT} of DFC-2 ranged from 0.19 to 0.39 μ g/ml against all tested *M. tuberculosis* strains. The antitubercular activity of DFC-2 was less effective than that of INH and RIF against drug-sensitive strains, but was far more effective against drug-resistant strains, especially against MDR and XDR.

Table 1. Sequences of primers used for qRT-PCR.

Gene symbol ^a	Gene description ^a	Locus tag ^a	Sense primer sequence (5'-3')	Antisense primer (5'-3')
De novo synthesis of fatty acids^b				
<i>fas</i>	Fatty acid synthase	Rv2524c	GATGTACCACGGCAATCT	GCAATGATATTCGGCAAGA
Fatty acid elongation				
<i>fabD</i>	Malonyl CoA-acyl carrier protein transacylase	Rv2243	AAACCGAGGGAATGTTGT	CTAGATCAGCGGCTTTCG
<i>acpM</i>	Meromycolate extension acyl carrier protein	Rv2244	TGTCACCTCAGGAAGAAATCATTG	ATCTCGGACGGCTCGATA
<i>kasA</i>	3-Oxoacyl-ACP synthase 1	Rv2245	ATCCCCGAGATCGACCTTG	CGAACGAGTTGTTGACTG
<i>kasB</i>	3-Oxoacyl-ACP synthase 2	Rv2246	ACACTGAATCTGGTAAAC	AATCCGAACGAGTTATTG
<i>fabG1</i>	3-Oxoacyl-ACP reductase FabG	Rv1483	TTCGGTCGAATGATATTCATAGGT	TGGAGGCTGCGTAGTTG
<i>inhA</i>	NADH-dependent enoyl-[ACP] reductase	Rv1484	ATGACAGGACTGCTGGAC	TACCCGTGCGATGTGAAA
<i>hadA</i>	(3R)-Hydroxyacyl-ACP dehydratase subunit HadA	Rv0635	ACGCGTGGTATTTTCGAG	CTTGTAGCCGAACACACAG
<i>hadB</i>	(3R)-Hydroxyacyl-ACP dehydratase subunit HadB	Rv0636	AGGTCGGAGACCAGCTTC	GAATCGGGTCAAGTACC
<i>hadC</i>	(3R)-Hydroxyacyl-ACP dehydratase subunit HadC	Rv0637	ATGGCGCTCAAGACC	GACAGCTCGGGCAAAC
<i>fabH</i>	3-Oxoacyl-[acyl-carrier-protein] synthase III	Rv0533c	GGGCTGCTCAGTGTCG	CCGGTTCGGGTGTAGAT
Synthesis of fatty/mycolic acid precursors				
<i>accD6</i>	Acetyl-/propionyl-CoA carboxylase subunit beta	Rv2247	GGGTGCACGCACATCGT	CCGAGATCTGCGGGATGTAG
<i>accD5</i>	Propionyl-CoA carboxylase subunit beta	Rv3280	CCGCTCGGCTCATTC	GACTCTTCCCTGCGTTTG
<i>accA3</i>	Bifunctional protein acetyl-/propionyl-CoA carboxylase subunit alpha AccA	Rv3285	TGGCTAGTCACGCCG	CGGATCACCCGCACT
Meromycolic acid functionalization				
<i>mmaA1</i>	Mycolic acid methyltransferase MmaA1	Rv0645c	CGAGCGTGACGATATGAC	GCAACCCACGTCGAGTA
<i>mmaA2</i>	Cyclopropane mycolic acid synthase CmaA	Rv0644c	CCAGGCAGCAGATGGT	AATCGTCGGAGGTTGG
<i>mmaA3</i>	Methoxy mycolic acid synthase MmaA3	Rv0643c	CGCCAAGCTGGACCTA	GGCACGCTTCATGACC
<i>mmaA4</i>	Hydroxymycolate synthase MmaA4	Rv0642c	GACCCGACCCGAACCTA	AGCTTGTCAGGTTGAGG
<i>cmaA1</i>	Cyclopropane mycolic acid synthase CmaA	Rv3392c	GCGCGGTGGAATAATACGAC	AAAGCACCGATGCTGACGA
<i>cmaA2</i>	Cyclopropane mycolic acid synthase	Rv0503c	TCAACGTCATCGGCTTG	CTGGATTGCGACCTCTTTT
<i>pcaA</i>	Cyclopropane mycolic acid synthase	Rv0470c	CGCGTTTCGAGCACTTC	GCACGATGGTGTGCAG
<i>umaA</i>	Mycolic acid synthase UmaA	Rv0469	ACGTCATCGGTATCACGCTC	GTCGACCTGTCCGTGAAC
Mycolic acid condensation				
<i>accD4</i>	Propionyl-CoA carboxylase subunit beta AccD	Rv3799c	ATCGTCGGCATCAACGACTC	CGGTCTGGATCGGCGAATAC
<i>pks13</i>	Polyketide synthase	Rv3800c	GGTCTCCTCGATGGTCTC	CTAAACAGCGTGCGTATG
<i>fadD32</i>	Long-chain-fatty-acid--AMP ligase FadD32	Rv3801c	GAAAAGTGGGCGAAGG	GAAGTCAGACCACAAGATGTC
<i>Rv2509</i>	Short-chain type dehydrogenase/reductase	Rv2509	CCCATCTCGATCCTGTGCG	CAGAAATCAAGATGCCGCCG
Chaperones/Heat shock				
<i>hsp</i>	Heat shock protein	Rv0251c	CCGAGATCGTCAAGGATGGC	TCTTTGTCTCCGGCGTCTTG
Ribosomal RNA				
<i>rrs</i>	16S ribosomal RNA	Rvnr01	GTGGCGAACGGGTGAGTAAC	ARGCATCCCGTGGTCCTATC

^aGene symbol, gene description, and locus tag as designated on the NCBI website (<http://www.ncbi.nlm.nih.gov/gene>)^bBold font indicates the functional category in the *M. tuberculosis* H37Rv genome.

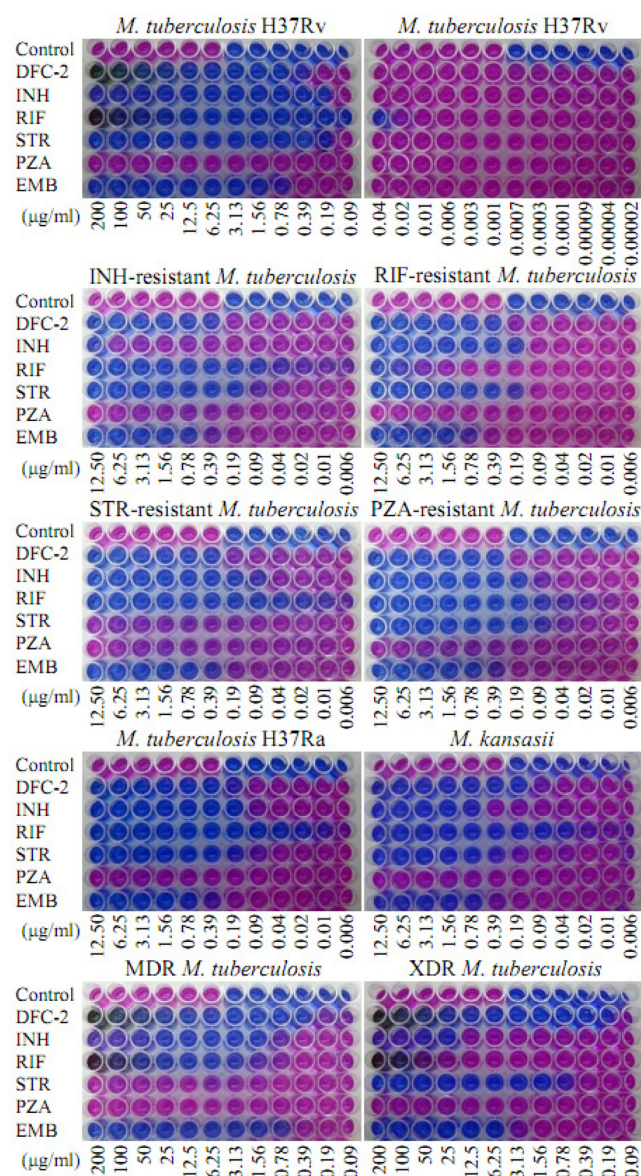


Fig. 2. In vitro antitubercular activity of DFC-2 and control drugs against *M. tuberculosis* H37Ra, H37Rv, drug-resistant strains, and non-tubercular mycobacterial strains. Resazurin is reduced to resorufin, which is pink when the mycobacteria grow.

Exploring DFC-2-Induced Alterations in Gene Expression in *M. tuberculosis* H37Rv

Microarray analysis, a powerful tool for studying the molecular basis of genetic interactions, makes it possible to examine the expression of thousands of genes simultaneously [19]. Transcriptional profiling of *M. tuberculosis* H37Rv was performed using 3,975 whole-genome microarrays in order to measure the effects of DFC-2 relative to a sample of

logarithmically growing *M. tuberculosis* H37Rv. When compared with the untreated control group, 1,786 ($10\times \text{MIC}_{\text{TDT}}$, 6 h) and 1,345 ($10\times \text{MIC}_{\text{TDT}}$, 24 h) genes were found to have altered expression levels by 2-fold or more. When the FC cutoff was raised to 5-fold, 291 ($10\times \text{MIC}_{\text{TDT}}$, 6 h) and 265 ($10\times \text{MIC}_{\text{TDT}}$, 24 h) genes were found to have altered expression levels in the DFC-2-treated group. In order to better understand the transcriptional response of *M. tuberculosis* to DFC-2, we adopted more specific functional classifications as previously described [20]. Hypergeometric distribution [21–23] was used to determine whether the enrichment of genes within a particular functional category in response to DFC-2 treatment was significant. Under >2 and 5 FC and $p < 0.05$ conditions, the most affected category was “lipid biosynthesis,” commonly seen at 6 and 24 h at $10\times \text{MIC}_{\text{TDT}}$ DFC-2 (Table 2). Moreover, genes involved in synthesis of fatty and mycolic acids were significantly downregulated ($p < 0.001$) in response to $10\times \text{MIC}_{\text{TDT}}$ DFC-2 under 2 and 5 FC cutoff (Table 3). A full list of microarray data is available as supplementary data.

To confirm the microarray results, we explored the gene transcript levels of 26 genes related to mycolic acid synthesis [24] among three groups of samples: control, $1\times$, and $10\times \text{MIC}_{\text{TDT}}$ DFC-2 by RT-PCR. In accordance with the protocols of microarray analysis, all mycolic acid synthesis-related genes were suppressed by, on average, 58.1% and 91.1% at $1\times$ and $10\times \text{MIC}_{\text{TDT}}$, respectively (Figs. 3A–3E). These results are quite a contrast to those of the heat shock protein gene (Fig. 3F), which is known to be upregulated under stress conditions such as exposure to antibiotics [25]. Among downregulated genes, mycolic acid condensation was the most overrepresented (Fig. 3G).

LC-MS/MS Analysis of Total Mycolate in DFC-2-Treated *M. tuberculosis* H37Rv

Our gene transcription results suggested that DFC-2's primary effect is to disrupt mycolic acid synthesis. Therefore, we tested whether DFC-2 affects mycolic acid synthesis in *M. tuberculosis*. In order to evaluate the inhibition of mycolic acid synthesis in response to DFC-2, we measured mycolic acid levels in *M. tuberculosis* over a period of 5 days. Interestingly, we found that DFC-2 triggered a reduction in mycolic acid production, showing a similar pattern to that of mycolic acid synthesis inhibitor INH (Fig. 4) [26]. The results of this experiment correspond well with those of a previous study, which reported that ursolic acid reduces mycolic acid levels [15]. The decrease in mycolic acid levels at a low, nontoxic concentration ($0.2\times$, $1\times \text{MIC}_{\text{TDT}}$) of DFC-2 without change in mycobacterial

Table 2. Number of genes (FC cutoff > 2 and 5) regulated in *M. tuberculosis* H37Rv by 1× and 10× MIC_{TDT} DFC-2 treatment for 6 and 24 h at 37°C, according to functional class.

Functional classification	6 h								24 h							
	2-fold				5-fold				2-fold				5-fold			
	1× MIC		10× MIC		1× MIC		10× MIC		1× MIC		10× MIC		1× MIC		10× MIC	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
I. Small-molecule metabolism																
A. Degradation (163) ^d	12	1	56	17	0	0	8	3	0	2	15	30	0	0	5	5
B. Energy metabolism (292)	13	6	80	61 ^b	0	0	14	9	1	0	38	77 ^b	0	0	6	14
C. Central intermediary metabolism (45)	3	1	10	7	0	0	2	0	0	0	9	9	0	0	1	3
D. Amino acid biosynthesis (95)	1	3	11	26 ^b	0	0	3	5	0	1	9	29 ^a	0	1	2	8 ^a
E. Polyamine synthesis (1)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F. Purines, pyrimidines, nucleosides, and nucleotides (60)	0	1	12	9	0	0	1	2	0	0	8	13	0	0	2	3
G. Biosynthesis of cofactors, prosthetic groups, and carriers (117)	3	1	19	20	0	0	4	4	1	0	9	34 ^a	0	0	4	6
H. Lipid biosynthesis (65)	1	3 ^a	12	23 ^c	0	0	0	8 ^c	1	0	5	21 ^a	0	0	0	6 ^a
I. Polyketide and nonribosomal peptide synthesis (41)	4	2	16	11 ^a	0	0	1	3	2	0	6	8	0	0	1	1
J. Broad regulatory functions (187)	13	2	85 ^c	21	0	0	12	5	1	2	42 ^c	25	0	0	7	5
II. Macromolecule metabolism																
A. Synthesis and modification of macromolecules (213)	6	2	41	66 ^c	0	0	9	15	3	0	26	31	0	0	8	3
B. Degradation of macromolecules (87)	4	4	25	19	0	0	5	2	0	0	10	19	0	0	3	3
C. Cell envelope (358)	14	1	88 ^c	55	2 ^c	0	3	6	3	0	30	76	0	0	6	9
III. Cell processes																
A. Transport/binding proteins (123)	1	1	30	23	0	0	1	3	0	0	12	29	0	0	3	5
B. Chaperones/heat shock (16)	3 ^a	0	6	2	0	0	3	0	1	1	8 ^c	5	0	0	4 ^c	1
C. Cell division (19)	1	1	2	4	0	0	0	1	0	0	0	6	0	0	0	1
D. Protein and peptide secretion (14)	1	0	2	6 ^a	0	0	1	1	0	0	0	6 ^a	0	0	0	2
E. Adaptations and atypical conditions (11)	0	0	1	4	0	0	0	1	0	0	0	7 ^b	0	0	0	0
F. Detoxification (22)	4 ^a	0	6	5	0	0	3	1	0	1	1	8	0	0	0	0
IV. Other																
A. Virulence (38)	2	0	10	3	0	0	1	1	0	0	2	13 ^a	0	0	0	4
B. IS elements, repeated sequences, and phage (134)	9	0	88	2	0	0	13 ^b	0	1	1	43 ^c	5	0	1	5	2
C. PE and PPE families (162)	8	2	57	25	0	0	10	4	6 ^b	1	27	29	0	0	7	6
D. Antibiotic production and resistance (14)	1	0	6	1	0	0	2	0	0	0	3	2	0	0	2	0
E. Bacteriocin-like proteins (3)	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0
F. Cytochrome P450 enzymes (22)	1	0	8	2	0	0	1	1	0	0	1	7	0	0	0	3 ^a
G. Coenzyme F420-dependent enzymes (3)	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1
H. Miscellaneous transferases (61)	2	2	15	7	0	0	2	1	3 ^a	0	9	14	0	0	2	5
I. Miscellaneous phosphatases, lyases, and hydrolases (18)	1	1	5	3	0	0	0	1	0	0	1	4	0	0	0	1
J. Cyclases (6)	0	0	4	0	0	0	1	0	0	0	0	0	0	0	0	0
K. Chelatases (2)	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
V. Conserved hypotheticals (914)	51	7	250	144	2 ^b	0	41	24	14	4	121	194	0	1	22	35
VI. Unknowns (587)	26	3	214 ^c	57	1	0	40	9	5	4	91 ^a	115	0	2	24 ^a	20

^a*p* < 0.05^b*p* < 0.01^c*p* < 0.001^dNumbers in parentheses indicate the total number of genes in the category in the *M. tuberculosis* H37Rv genome.

Table 3. Number of genes (FC cutoff > 2 and 5) regulated in lipid biosynthesis by 1× and 10× MIC_{TDT} DFC-2 treatment for 6 and 24 h at 37°C, according to functional class.

Functional classification	6 h								24 h							
	2-fold				5-fold				2-fold				5-fold			
	1× MIC		10× MIC		1× MIC		10× MIC		1× MIC		10× MIC		1× MIC		10× MIC	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
I. Small-molecule metabolism																
H. Lipid biosynthesis																
10. Synthesis of fatty and mycolic acids (26) ^d	0	2 ^a	5	13 ^c	0	0	0	5 ^c	0	0	2	7	0	0	0	2
20. Modification of fatty and mycolic acids (14)	1	0	4	3	0	0	0	0	1	0	2	5	0	0	0	1
30. Acyltransferases, mycolyltransferases, and phospholipid synthesis (25)	0	1	3	7	0	0	0	3 ^a	0	0	1	9	0	0	0	3

^a*p* < 0.05

^b*p* < 0.01

^c*p* < 0.001

^dNumbers in parentheses indicate the total number of genes in the category in the *M. tuberculosis* H37Rv genome.

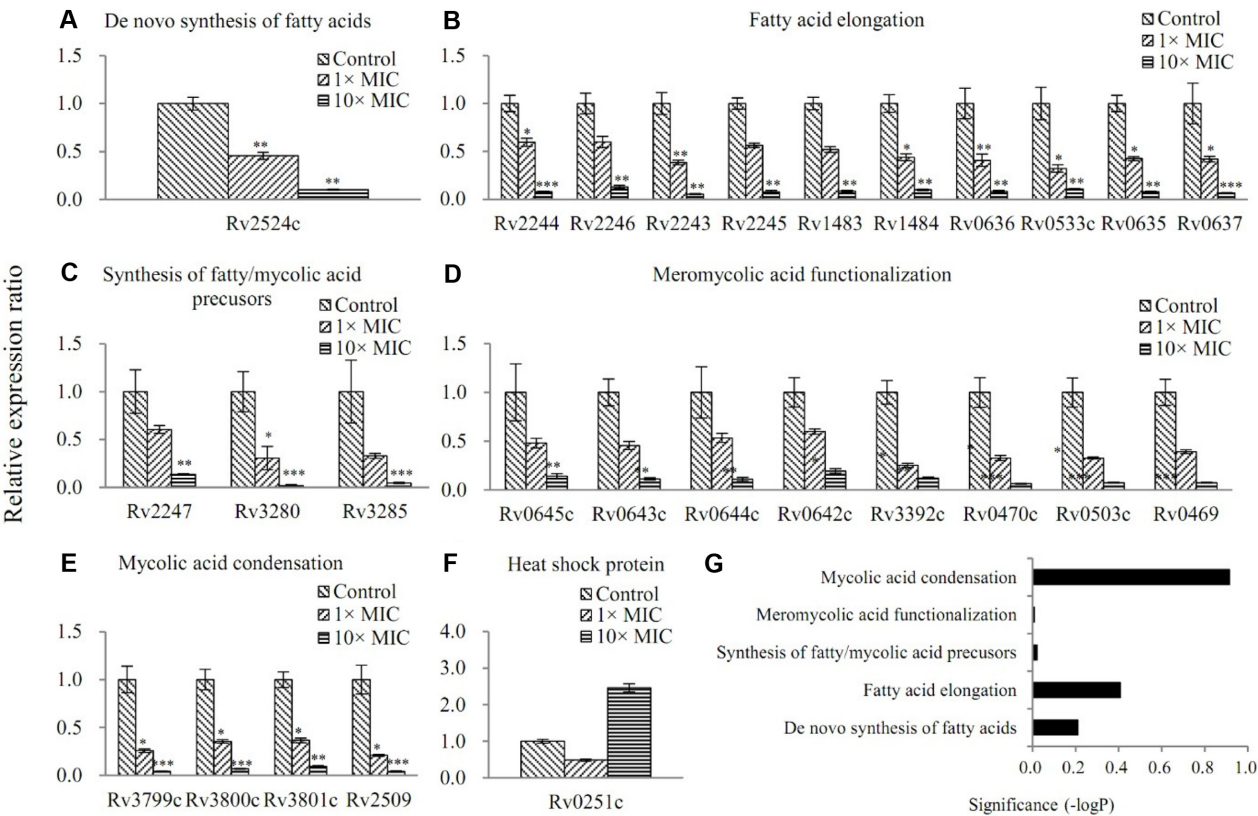


Fig. 3. Changes in mRNA levels of *M. tuberculosis* genes involved in (A–E) mycolic acid synthesis and (F) heat shock protein during exposure to 1× and 10× MIC_{TDT} DFC-2 for 6 h at 37°C.

Differences between DFC-2 effects and control, such as 1× and 10× MIC DFC-2 vs. control group, were assessed using a two-tailed Student's *t*-test for statistical significance. Values shown are the mean ± SEM of triplicate experiments (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). (G) The key functional categories of mycolic acid synthesis-related genes identified as significantly differently expressed (*p* < 0.05) in 10× MIC_{TDT} DFC-2 treatments. The x-axis represents the logarithms of the hypergeometric-based probabilities for the significance of the category. Black bars represent functional categories for the 26 significantly downregulated genes. MIC means the minimum inhibitory concentration and MIC_{TDT} means the MIC value measured by the tube dilution technique (TDT).

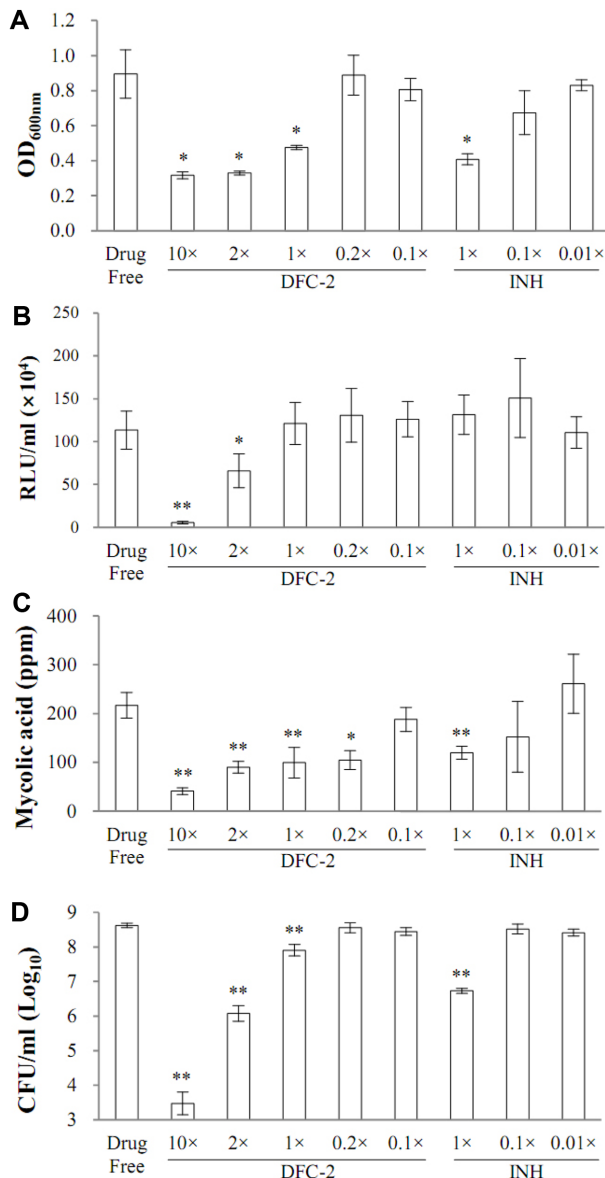


Fig. 4. Effects of DFC-2 and isoniazid (INH) on *M. tuberculosis* H37Rv growth after 5 days at 37°C.

(A) OD_{600nm} value, (B) cell viability, (C) mycolic acid levels, and (D) CFU are shown. Experiments were carried in triplicate and repeated at least three times, and the data are given as the mean values and SEM. *Statistical significance vs. drug-free control using unpaired Student's *t*-test (**p* < 0.05, ***p* < 0.01). TDT (tube dilution technique) was used to determine the minimum inhibitory concentration (MIC) of DFC-2 (1× MIC, 3.7 µg/ml) and INH (1× MIC, 0.37 µg/ml) in this test.

viability strongly suggests that DFC-2 is highly effective in reducing mycolic acid levels in *M. tuberculosis* [15].

Observation under Electron Microscopes

The inhibitory properties of DFC-2 on the synthesis of

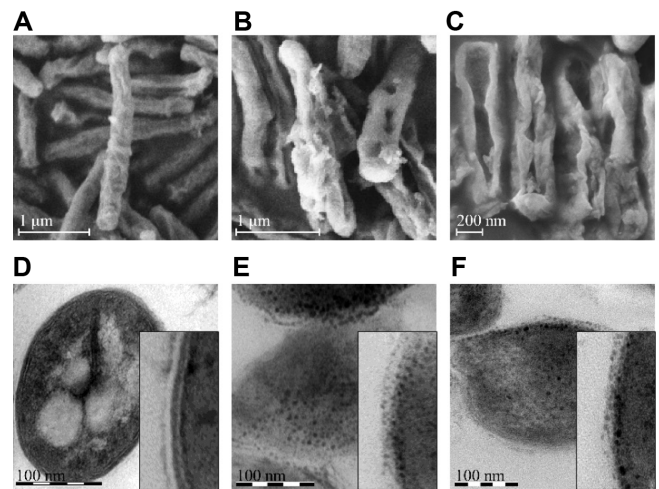


Fig. 5. Electron microscopy of DFC-2-treated cells.

Scanning electron micrographs. (A) Control, (B) 1× MIC_{TDT} INH, (C) 1× MIC_{TDT} DFC-2 treated cells. (D–E) Transmission electron micrographs. (D) Control, (E) 1× MIC_{TDT} INH, (F) 1× MIC_{TDT} DFC-2 treated cells.

mycolic acid prompted us to investigate whether DFC-2 has an effect on the cell envelope of *M. tuberculosis* cells. The observations were compared with that of INH, a mycolic acid synthesis inhibitor. *M. tuberculosis* cells in the control culture have a smooth surface with a well-defined rigid rod shape (Figs. 5A and 5D). The 1× MIC_{TDT} INH and DFC-2 exposed cells displayed almost similar morphological changes (Figs. 5B and 5C). After 3 days of exposure, the cells appeared ragged with tiny holes, and amorphous masses of cells were also observed. The TEM image of the 1× MIC_{TDT} INH and DFC-2-treated cells on day 3 showed the fully lysed cell envelope with the extrusion of intracellular contents (Figs. 5E and 5F). This effect of INH and DFC-2 on the cellular morphogenesis of *M. tuberculosis* is consistent with the results presented in a previous study [27]. This result strongly supports the hypothesis of a direct effect of DFC-2 on mycolic acid biosynthesis similar to that of INH.

Comparison of Gene Expression Profiles between INH, ETH, and DFC-2

Mycolic acids are long-chain branched fatty acids that represent the viability, drug resistance, and cell wall integrity of the mycobacteria [28], whose biosynthesis involves five distinct stages: (i) the synthesis of α -branch fatty acid by FAS-I, (ii) the synthesis of meromycolic acids by FAS-II, (iii) the introduction of functional groups to the meromycolate chain, (iv) the condensation of the α -branch and the meromycolate to produce mycolic acids, and (v) the transfer of mycolic acids to arabinogalactan [29]. In

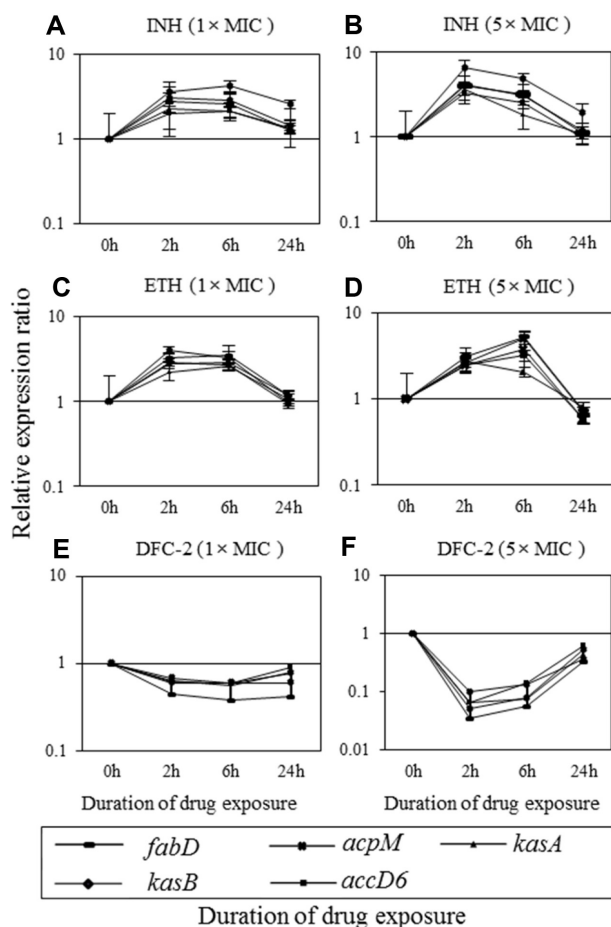


Fig. 6. Response of the *kas* operon to treatment with INH (A, B), ETH (C, D), and DFC-2 (E, F), as measured by RT-PCR. The ratio between the number of cDNA copies detected in each sample relative to the drug-free control at 1× MIC (A, C, E) and 5× MIC (B, D, F) is represented. INH, isoniazid; ETH, ethionamide.

order to evaluate if the mycolic acid synthesis inhibition and cell envelope disruption effects of DFC-2 were coupled with the decrease in transcription of the mycolic acid synthesis-related genes or primary target for DFC-2 in mycolic acid biosynthesis, we went on to perform RT-PCR analysis of *kas*/FAS-II operon genes (*fabD*-*acpM*-*kasA*-*kasB*-*accD6*) that are considered a hallmark of mycolic acid synthesis [24, 30–32], in response of *M. tuberculosis* to treatment with the DFC-2 and mycolic acid biosynthesis inhibitors (INH, ETH) whose target is *InhA* [33]. Results showed an increase in the expression of the *kas*/FAS-II operon genes in response to INH or ETH treatment for 2 h, followed by a slow decrease in their expression following 6 and 24 h treatment. Meanwhile, DFC-2 treatment showed a dramatic reduction for 2 h, and the degree of decline slowed down at 24 h (Fig. 6). The response to INH was

similar to that of a previous study where it showed an increase in the expression of the *kas*/FAS-II operon genes and the effect of DFC-2 was similar to that of another mycolic acid inhibitor, triclosan [32], which indicates that both INH and DFC-2 potentially inhibit mycolic acid biosynthesis, but their primary targets are different.

In summary, our results indicate that DFC-2 inhibits the growth of *M. tuberculosis*, which may be the result of its effects on mycolic acid biosynthesis. However, the mechanism by which DFC-2 inhibits mycolic acid biosynthesis has yet to be understood. Thus, further studies will be required to investigate the mechanism of action of DFC-2 against *M. tuberculosis* at the molecular level.

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