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Research paper

Design, synthesis and evaluation of covalent inhibitors of DprE1 as antitubercular agents

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ABSTRACT

Decaprenylphosphoryl-β-D-ribose 2′-oxidoreductase (DprE1) is a promising drug target for the development of novel anti-tubercular agents, and inhibitors of DprE1 are being investigated extensively. Among them, the 1,3-benzothiazinone compounds such as BTZ043, and its closer congener, PBTZ169, are undergoing clinical studies. It has been shown that both BTZ compounds are prodrugs, the nitro group is reduced to nitroso first, to which an adjacent Cys387 in the DprE1 binding pocket is covalently bound and results in suicide enzyme inhibition. We figured that replacement of the nitro with an electrophilic warhead would still achieve covalent interaction with nucleophilic Cys387, while the required reductive activation could be circumvented. To test this hypothesis, a number of covalent inhibitors of DprE1 were designed and prepared. The compounds inhibitory potency against DprE1 and anti-tubercular activity were investigated, their chemical reactivity, formation of covalent adduct between the warhead and the enzyme was demonstrated by mass spectrometry.

Tuberculosis (TB), the disease caused primarily by *Mycobacterium tuberculosis* bacillus, has been torturing mankind since the ancient times. With the invention of isoniazid, pyrazinamide, rifamycin and ethambutol in the 1950s and 1960s, the disease was considered curable. Although the number of TB cases has been decreasing globally during the last 20 years, the last report by World Health Organization still estimates 10 million new cases and 1.45 million deaths in 2018 [1]. In addition, surveillance data emphasize the ongoing problem of multidrug resistant TB [1], related to poor adherence to the professional prescription, or co-morbidities resulting in the compromised immune response. The occurrence of drug resistance and the need to make the long and demanding TB treatment more efficient, leads to continuous efforts to develop novel drugs against TB.

One of the most promising targets for the development of new antituberculosis agents is decaprenylphosphoryl-D-ribose 2′-oxidoreductase (DprE1) [2]. DprE1 is a highly conserved FAD-containing enzyme, which, along with its downstream NADH-dependent reductase DprE2, catalyzes the conversion of decaprenylphosphoryl-β-D-ribofuranose (DPR) to decaprenylphosphoryl-β-D-arabinofuranose (DPA). DPA is essential for the synthesis of the mycobacterial cell wall component arabinan [3], and the sole donor substrate for a series of membrane-embedded arabinosyltransferases including the ethambutol targets EmbA, EmbB, and EmbC [4]. Recently, the periplasmic localization of DprE1 was confirmed, which further substantiates the vulnerability of DprE1 as a promising antitubercular drug target [5].

The first identified class of DprE1 inhibitors were nitro-

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benzothiazinones (BTZs), with compound BTZ043 (Fig. 1) [2] as the lead. The minimal inhibitory concentration (MIC) value, for the next generation nitro-benzothiazinone, PBTZ 169 (Fig. 1), is as low as 1 ng/mL against *Mycobacterium tuberculosis* [6]. This value is 20 folds lower than that of the well known first line drug isoniazid. Structure-activity relationship study confirmed that the 8-nitro group is critical for both enzymatic and cellular activity. Upon binding to DprE1, the nitro group is reduced to a nitroso and that specifically forms a covalent adduct with the Cys387 residue in the active site of DprE1[7,8].

Recently, a boost to develop covalent drugs has occurred, examples of purposely designed covalent inhibitors are currently approved or at advanced stage of clinical trials [9]. Compared to the non-covalent counterparts, covalent drugs show strong and long acting interaction property that allows better pharmacologic response at a lower concentration [10]. To engineer a covalent drug, the protein/inhibitor interacting pocket features the existence of a nucleophilic residue, such as Ser/Thr, Lys, Met or Cys. Accordingly, the inhibitor molecule bears an electrophilic warhead, or a moiety that could be metabolically activated to the electrophilic part. Typical warhead was observed as a Michael addition acceptor [11], or a cyano group [12]. In DprE1, the presence of Cys387 in the proximity of the BTZ binding site provides the opportunity to achieve selectivity by covalent targeting with electrophilic inhibitors [13]. In this study, we investigated three types (**A**, **B**, and **C**) of BTZ derived compounds, with nitro group replaced by electrophilic warheads (Fig. 2). Compared to the reported prodrug PBTZ169, our designed compounds were expected to inhibit DprE1 through formation of covalent adduct without going through the reductive activation step.

1. Results and discussion

1.1. Chemistry

The designed type **A** and type **C** compounds were synthesized following the synthetic route shown in Scheme 1.

PBTZ169 (**1**) was prepared according to the published procedure [6]. Next, reduction of the nitro group to amino was achieved using iron and ammonium chloride. The following Sandmeyer reaction proceeded successfully using *tert*-butyl nitrite/$NaNO_2$ in combination with copper halides, and converted the aromatic amino group to the corresponding halides (**C01**, **C02** and **C03**). Compound **C03** further served as a common intermediate for the preparation of compounds **C04** and **A01-A07**. Specifically, reaction of **C03** with zinc cyanide using tetrakis (triphenylphosphine)palladium (0) as catalyst gave compound **C04**. Reaction of **C03** with methyl acrylate, acrylamide, *N*-methacrylamide, *N*,*N*-dimethyl acrylamide, acrylonitrile, methyl propiolate, 2-vinylpyridine provided **A01-A07**, accordingly. Hydrogenation of **A01**, **A05** and **A02** using pd/C as catalyst furnished **D01**, **D02** and **D03**. All double bond configuration was assigned as *trans*-based on the coupling constant in the ^{1}HNMR [14].

The designed type **B** compounds were prepared following the route shown in Scheme 2.

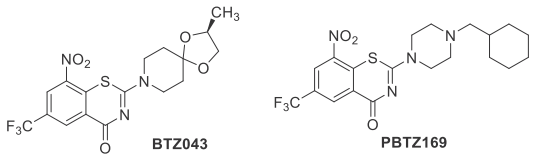


Fig. 1. Reported 1,3-benzothiazinone type DprE1 inhibitors BTZ043 and PBTZ169.

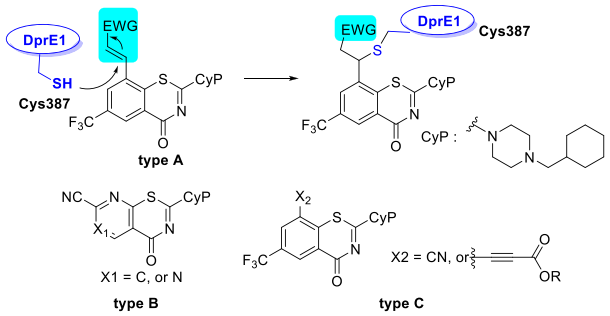


Fig. 2. Design rationale for covalent inhibitors of DprE1.

As shown in Scheme 2, The cyano containing compounds **B01**, **B02** and **B03** were prepared from commercial available starting material 2,6-dichloronicotinic acid or 2,4-dichloropyrimidine-5-carboxylic acid. The carboxylic acid was first transformed using oxalyl chloride to acyl chloride **3a**/**3b**, which was treated with ammonium thiocyanate to provide isothiocyanate **4a**/**4b**. Compound **B02** was successfully prepared through one more step reaction between **4b** and 1-(cyclohexylmethyl)piperazine. While reaction of **4a** with 1-(cyclohexylmethyl)piperazine gave intermediate **5** instead of the desired cyclization product, further treatment of **5** with triethylamine in toluene under reflux condition provided intermediate **6.** Finally, compound **B01** was prepared through reaction of **6** with zinc cyanide using palladium catalyst.

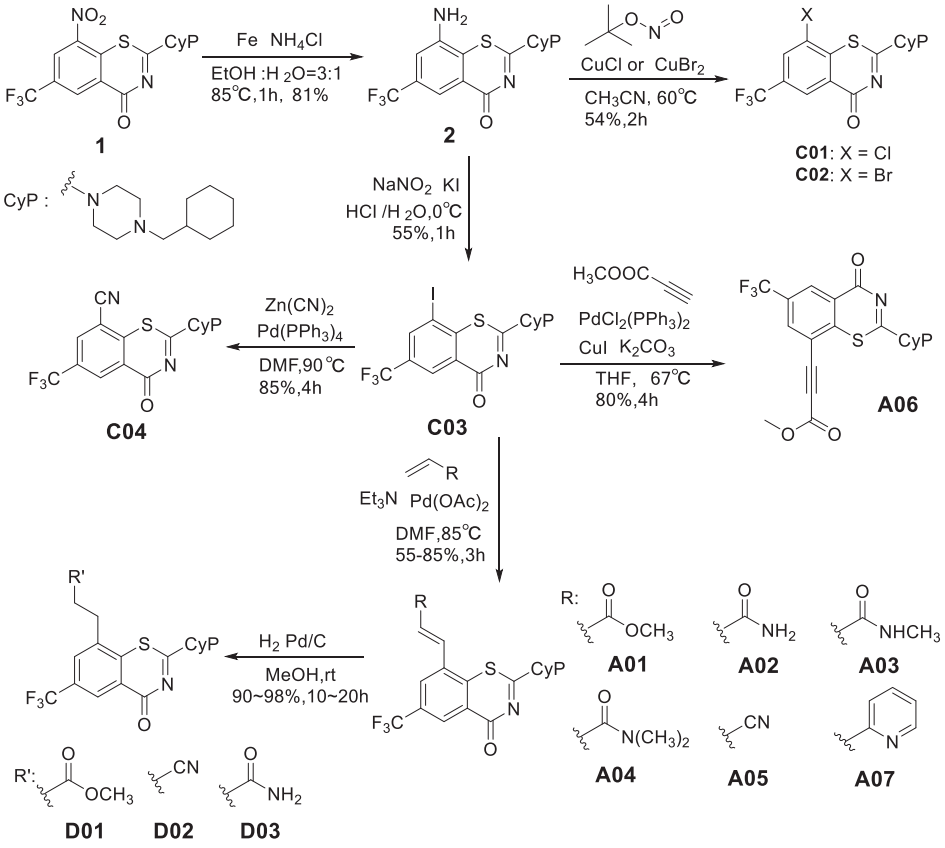
1.2. Biological evaluation and chemical stability study

The prepared compounds were evaluated for their inhibitory activity against both DprE1 and *M. tuberculosis* H37Rv (ATCC 25618). The results are shown in Table 1.

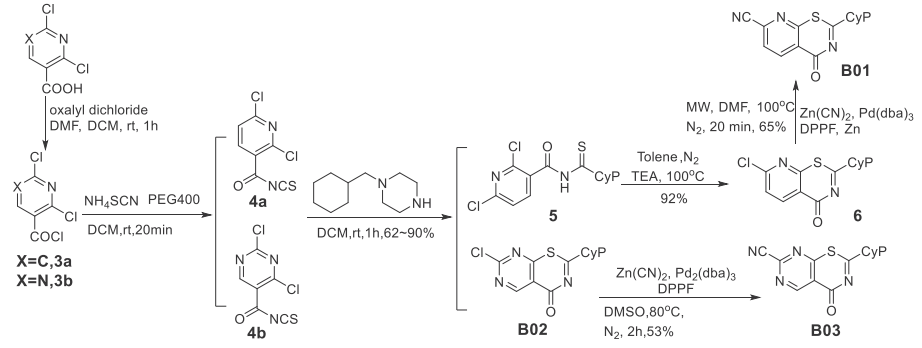
Overall, five out of seven type **A** compounds displayed good DprE1 inhibitory activity, with **A02**, **A03** and **A06**, in which nitro group replaced by acrylamide, *N*-methacrylamide and methyl propiolate exhibited IC_{50} less than 0.4 μM; and **A01** and **A04** (methyl acrylate and *N*,*N*-dimethyl acrylamide, respectively) displayed similar IC_{50} 1.25 μM against DprE1. It was noted that low activity is observed for compound **A07** (no inhibitory activity at concentration up to 20 μM), considering this compound bears more bulky pyridyl (**A07**), the result may suggest that the binding pocket for the BTZ scaffold at 8-position is space restricted, consistent with previously reported study [15]. It is surprising to notice compound **A05**, with cyano group as warhead, showed dramatic loss of enzyme inhibition activity. Subsequent chemical reactivity study with methyl thioglycolate and stability investigation in PBS buffer revealed **A05** decomposition in the assay solution, explaining the observed low biological activity.

Inconsistent with the enzyme activity, compounds with high inhibitory potency against DprE1 did not display good cellular activity. As shown in Table 1, all type **A** compounds displayed low *anti*-Mtb H37Rv activity, with MIC values mostly higher than 20 μM. This result suggested the compound poor ability to penetrate the mycomembrane and implement the inhibitory ability.

To validate the covalent inhibitor design rationale, we investigated the compound reactivity toward a nucleophile like methyl thioglycolate (MT) [16]. The result is shown in Table 2. Indeed, except **A03** and **A07**, all other tested compounds show reactivity with electrophile, and covalent adducts with MT were detected as subjecting to mass analysis. The most reactive **A05** had a half life 0.2 h. Further stability study in pH7.4 PBS buffer recorded a half life about 25 min, suggesting the possible decomposition process in the assay solution, which also explains the observed lack of DprE1 and



Scheme 1. Synthetic route for compounds **A01-A07, C01**—**C04** and **D01-D03**.



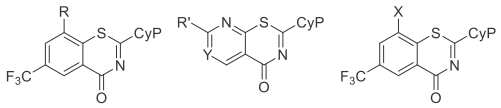
Scheme 2. Synthetic route for compound **B01**, **B02** and **B03**.

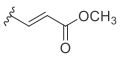
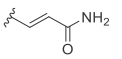
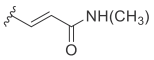
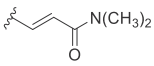
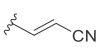
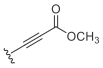
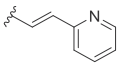
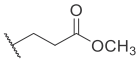
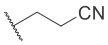
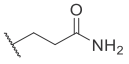
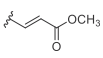
anti mycobacterial activity. By comparison, compound **A06** (methyl propiolate), **A01** (methyl acrylate), **A02** (acrylamide), **A04** (*N*,*N*-dimethyl acrylamide) displayed stability improvement, half life between 1.0 h and 24 h in the presence of MT. Although **A07** displayed low chemical reactivity toward MT, its weak DprE1 inhibitory activity was more likely due to the intolerance of the bulky pyridyl group in the enzyme binding pocket. Additionally, our detected MT adduct peak for each compound correlates well with the calculated mass (supporting information).

With the 7-cyano warhead, compounds **B01** and **B03** were expected to act as covalent DprE1 inhibitors. Unfortunately, both compounds lost DprE1 activity. Compound **B02**, prepared as the reaction intermediate for **B03**, also shown low DprE1 activity. Taking together, the results suggest only phenyl other than other hetero aromatic rings could be accommodated in the binding pocket, which is in agreement with the previously reported study [2]. Alternatively, it could be possible that 7-substitution on the phenyl ring was deleterious for enzyme activity. With low DprE1 inhibition capability, the type **B** compounds demonstrated weak (**B03**, MIC 6.7 μM) or no (**B02**, MIC>20 μM) antibacterial activity (Table 1). We confirmed that **B02** could form covalent adduct with MT, and the half life is about 4 h.

During the process of preparation type **A** compounds, the halogen substituted **C01**, **C02** and **C03** were readily obtained. Although these compounds would not act through a covalent inhibition manner as PBTZ169 does, it was interesting to justify how well the 1,3-benzothiazin-4-one scaffold exerts its enzymatic inhibition activity in a non-covalent binding mode. Thus, compounds **C01**—**C03** were examined for their DprE1 inhibition activity, and micromolar IC_{50}s were recorded (Table 1), which confirmed the

Table 1
Inhibition of DprE1 by prepared compounds and their activity against *M. tuberculosis* H37Rv strain.



Compd	core	R(X)	IC_{50}, DprE1 (μM)	MIC(μM, *Mtb* H37Rv)
A01	A		1.24 (±0.30)	>20.00
A02	A		0.20 (±0.04)	20.00 (±1.4)
A03	A		0.39 (±0.11)	20.00 (±1.56)
A04	A		1.25 (±0.20)	>20.00
A05	A		[a]	>20.00
A06	A		0.14 (±0.02)	10.00 (±0.26)
A07	A		[a]	>20.00
B01	B	Y=C, 	7.72 (±0.75)	>12.00
B02	B	Y=N, 	>20.00	>20.00
B03	B	Y=N, 	>20.00	6.73 (±0.44)
C01	C		0.92 (±0.13)	0.03 (±0.01)
C02	C		1.84 (±0.30)	0.16 (±0.02)
C03	C		2.35 (±0.25)	0.63 (±0.04)
C04	C		1.70 (±0.84)	0.16 (±0.10)
D01	C		5.40 (±0.77)	>9.00
D02	C		13.2 (±1.77)	>9.00
D03	C		16.7 (±1.95)	>9.00
1(PBTZ169)			0.01 (±0.001)	0.011 (±0.02)

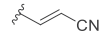
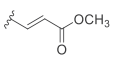
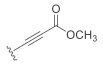
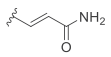
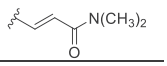
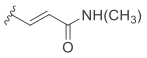
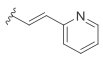
"a":compound shows less than 70% inhibitory activity at 20 μM.

significance of the nitro group for DprE1 activity in PBTZ169. The cyano is an apparent covalent warhead and drugs incorporated cyano were discovered in several precedents [17], thus, compound **C04** was prepared and studied. It was found that **C04** displayed IC_{50} 1.7 μM for the enzyme. However, all type **C** compounds did retain significant *M. tuberculosis* potency, with MIC 0.03 μM for **C01**, 0.16 μM for **C02** and 0.63 μM for **C03**. Compared to PBTZ169 (MIC 0.01 μM), the antibacterial activity decrease for **C01** was not as pronounced as the enzyme inhibition activity loss. Particularly, it was worthy to say that the antibacterial potency of type **C** compounds were superior to the current first-line drug isoniazid (INH, MIC 2.5 μM).

1.3. Type a compounds bind covalently to DprE1

To confirm the designed type **A** compounds could covalently bind to DprE1, **A01** and **A02** were selected as representative and co-incubated with the enzyme. As a control, DprE1 was incubated with only the solvent DMSO. The mixture was then dialyzed and subjected for mass analysis. As shown in Fig. 3, DprE1 protein

Table 2
Compound reactivity toward methyl thioglycolate (MT).

Compd	core	R(X)	Reactivity with MT (TLC monitor)	Half life $t_{1/2}$ (h)	Calculated covalent adduct mass/detected adduct peak
A05	A			0.2	568.7/568.6
A01	A			1.0	601.7/601.6
A06	A			1.2	599.7/599.6
B02	B			4	449.6/449.7
A02	A			10	586.7/586.6
C04	C			12	542.6/542.6
A04	A			>48	614.7/614.6
A03	A				no
A07	A				no

incubated with DMSO showed a MW of 50,940 Da. By contrast, the protein showed a MW of 51,430 Da after incubation with **A01**, and 51,390 Da when incubated with **A02**, thus an increase in molecular weight of 490 ± 20 or 460 ± 20 Da, respectively, was observed, which supports the covalent adduct formation between DprE1 and the compounds.

Next, to confirm that the covalent bond is formed with the Cys387, the modified protein was subjected to trypsin digestion, and the peptide fragments were analyzed. The LC-MS profile of the protein incubated with **A01** showed a peak with a retention time of 43.27 min, that was absent in profile of the DprE1 alone (Fig. S1, panel B, supporting information). The fragmentation of the peptide showed a sequence compatible with ^{387}-CVDFP-^{391}, with the compound forming an adduct with Cys387 (Fig. S2, top panel). Similarly, the LC-MS profile of the protein incubated with **A02** showed an additional peak with a retention time of 29.33 min (Fig. S1, panel C, supporting information) with the peptide compatible with the sequence ^{385}-NICVDFPIK-^{393}, with the compound bound to the Cys387 (Fig. S2, bottom panel). Thus, the data confirm that the compounds react with the Cys387 of DprE1, forming a covalent adduct.

To further examine the covalent inhibitor design rationale, the double bond in the warhead in the A-type of compounds **A01**, **A05** and **A02** was reduced to single bond and three type **D** compounds (**D01**-**D03**) were obtained. Compared to the corresponding double bond counterparts, **D01** lost its inhibitory activity about 5 times, **D02** increased its inhibitory activity and **D03** displayed dramatic loss of activity against DprE1 enzyme. Our efforts to identify the covalent adduct between **D01** and DprE1 enzyme failed, which suggests that covalent bond was not formed in this case, as expected.

1.4. Metabolic labeling of M. tuberculosis indicates inhibition of DprE1 by the target compounds in the cells

Despite promising inhibitory activities of the selected compounds from A-series against DprE1 enzyme, their MIC values for *M. tuberculosis* H_{37}Rv were rather high (Table 1). Therefore, we were interested if these molecules target DprE1 in the cells. For this experiment we chose compounds **A01**, **A02**, **A03** and **A06** and examined their effects in *M. tuberculosis* H37Rv by metabolic labeling, as described [18]. The cultures were radiolabeled in three different conditions: (i) for 24 h with [^{14}C] acetate added along with the drugs at 100 × MICs, (ii) for 24 h with [^{14}C] acetate added along with the drugs at 10 × MICs, (iii) for 24 h at 10 × MICs with [^{14}C] acetate added after 24 h of pre-treatment with the drug. The compound **A01**, for which MIC was found to be > 20.00 μM was tested at 0.2 mM and 2 mM concentration. The lipids were extracted from the radiolabeled cells and analyzed by thin-layer chromatography (TLC). As shown in Fig. 4, addition of the control inhibitors, BTZ043 and PBTZ169 caused accumulation of trehalose dimycolates (TDM) and trehalose monomycolates (TMM) in comparison with the control cells. Inhibition of DprE1 by these drugs results in restriction of synthesis of arabinan chains available for the attachment of mycolic acids. Under these conditions, TMM, the donor of mycolic acids for their deposition on arabinogalactan, serves as an acceptor of surplus mycolic acids and TDM is produced. Consequently, increased amounts of TMM and TDM can be considered as a "signature" for inhibitors targeting arabinogalactan portion of mAGP. This phenotype was clearly produced by the control drugs BTZ043 and PBTZ169 at 100 × MIC, while the effects of the tested compounds from A-series were not so profound. For compound **A06** we observed just overall decrease in [^{14}C] acetate incorporation into lipids under these conditions. However, at 10 × MIC and longer incubation in the presence of the tested compounds, we can see the expected phenotype, particularly for the compounds **A01**, **A02** and **A06**. The results of this experiment suggest that these compounds target DprE1 in *M. tuberculosis* H37Rv. Likewise, compounds **C01** and **C02**, which were examined by the same approach, appear to target DprE1 based on the lipid profiles obtained from the radiolabeled cells, although covalent binding is not expected in this case.

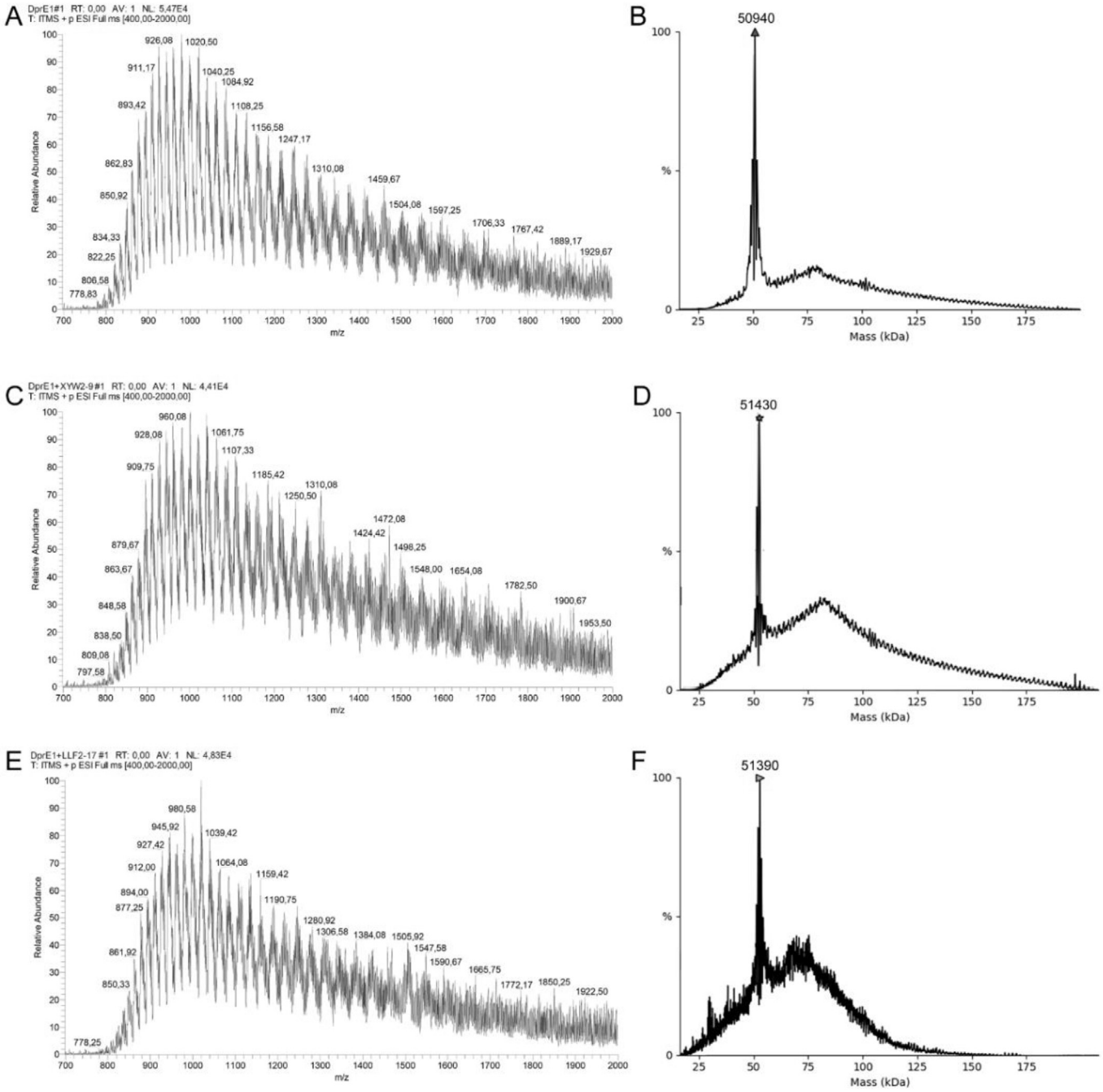


Fig. 3. Mass spectrometry analysis of DprE1 and DprE1-**A01**/DprE1-**A02** complexes demonstrates the formation of a covalent adduct between the protein and the compound. A) ESI-MS profile of DprE1; B) deconvolution of the spectrum in panel A; C) ESI-MS profile of DprE1 after incubation with **A01**; D) deconvolution of the spectrum in panel C. E) ESI-MS profile of DprE1 after incubation with **A02**; F) deconvolution of the spectrum in panel E.

2. Conclusion

In summary, we have designed and prepared a number of covalent inhibitors of DprE1. Biochemical evaluation of these compounds was performed. We validated the concept of selective targeting of the cysteine of the DprE1 active site through incorporation of a warhead at the 8 position of 1,3-benzothiazin-4-one scaffold. Compared to the reported nitro BTZ inhibitors, our designed covalent inhibitors circumvent the required reductive activation step. Nonetheless, delicate balance for the compound stability and reactivity towards a nucleophile is critical for practical application of these covalent inhibitors.

3. Experimental section

All commercially available reagents and solvents were used as received. All reactions were monitored by thin layer chromatography (TLC). Compounds were detected by ultraviolet light (UV) absorption at either 254 or 365 nm. TLC was performed on silica HSGF254 plates. All final products were characterized by ^{1}H NMR, ^{13}C NMR and MS analyses.^{1}H NMR and ^{13}C NMR spectra were measured on an INOVA-400 MHz or Agilent DD2-600 MHz spectrometer and referenced to TMS. Analysis of sample purity was performed on SHIMADZU LC-20AD high performance liquid chromatography (HPLC) system. HPLC conditions were as follows:

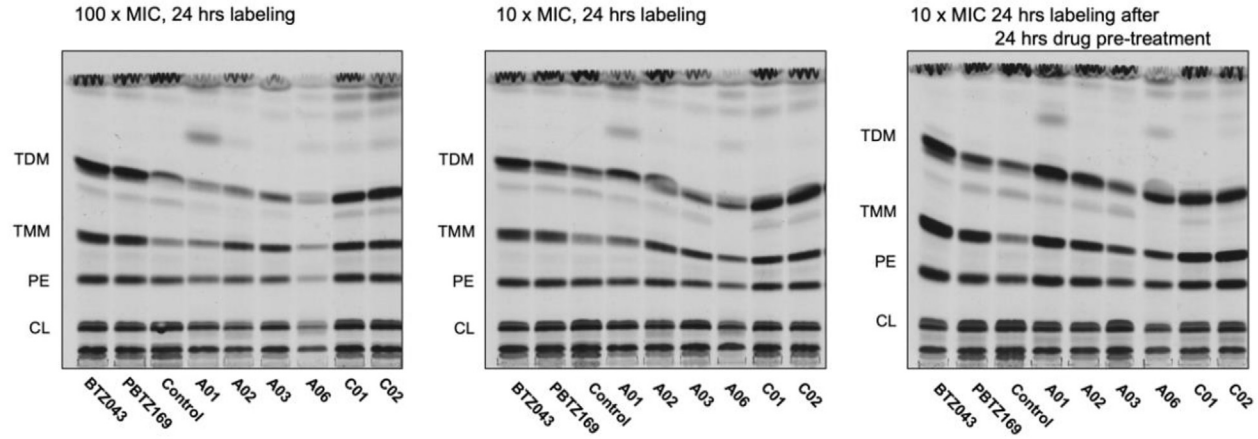


Fig. 4. Evaluation of the effects of the prepared compounds by metabolic radiolabeling. TLC analysis of the lipids from radiolabeled *M. tuberculosis* H37Rv. Mycobacteria were incubated with [^{14}C]-acetate and BTZ043, PBTZ169 or compounds **A01, A02, A03, A06**, **C01** and **C02** at 100 × MIC (left panel) or 10 × MIC concentration for 24 h (middle panel). Inhibitors were added along with the label (left and middle panels) or 24 h prior to label addition (right panel). TMM, trehalose monomycolates; TDM, trehalose dimycolates. PE, phosphatidyl ethanolamine, CL − cardiolipin. TLC analysis was performed on Silica Gel plates in $CHCl_3/CH_3OH/H_2O$ (20:4:0.5).

solvent A: water, solvent B: MeOH, and flow rate 1.0 mL/min. Compounds were eluted with a gradient from 10 to 100% MeOH/water in 30 min, and purity was determined by the absorbance at 254 nm and 300 nm. All tested compounds have a purity of ≥98% before sending for biological test.

3.1. Preparation of intermediate 2

To a solution of compound **1** (116 mg, 0.25 mmol) in EtOH/water (3:1, 8 mL) was added NH_4Cl (0.7 mg, 0.013 mmol) and Fe powder (142 mg, 2.54 mmol). The resulting mixture was heated at reflux and stirred for 1 h. After cooling to room temperature, the mixture was filtered through a pad of diatomaceous earth, which was then washed with more methanol (MeOH, 10 mL × 3). The filtrate was concentrated under reduced pressure and the resulting aqueous fraction was extracted with dichloromethane (DCM, 20 mL × 3). The combined extracts were washed with brine, dried (Na_2SO_4), filtered, and concentrated. The crude residue was purified by flash column chromatography on silica gel with DCM: MeOH (90:1) to afford intermediate **2** (88 mg, yield 82%) as a white solid: $R_f = 0.2$, CH_2Cl_2/MeOH: 90:1.

3.1.1. 2-(4-(Cyclohexylmethyl)piperazin-1-yl)-8-iodo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-4-one (C03)

To intermediate **2** (100 mg, 0.23 mmol) suspended in water (20 mL) at 0−5 °C was added dilute HCl (10%, 2 mL) and $NaNO_2$ (24 mg, 0.35 mmol, 1.5 equiv.) solutions successively, and the resulting reaction mixture was stirred for 1 h to complete the diazotization process. Then KI (78 mg, 0.47 mmol, 2.0 equiv.) was slowly added, and the reaction was stirred overnight at room temperature. The mixture was filtered and washed with distilled water (10 mL) and extracted with EtOAc (10 mL × 3), and the combined organic layer was washed with 10% aqueous solution of Na_2SO_3 (15 mL) and then dried over anhydrous Na_2SO_4, filtered, and concentrated. The crude residue was purified by flash column chromatography on silica gel with ethyl acetate (EtOAc) and petroleum ether (PET), EtOAc-PET (1:1) to afford compound **C03** (70 mg, yield 56%) as a yellow solid: $R_f = 0.2$, EtOAc:PET (1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.75 (s, 1H), 8.18 (s, 1H), 4.43 (br, 2H), 4.09 (br, 2H), 2.62 (s, 4H), 2.26 (s, 2H), 1.81−1.75 (m, 6H), 1.71 (s, 1H), 1.33−1.21 (m, 2H), 0.92−0.87 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.4, 161.7, 141.0, 138.3(d, $J = 3.0$ Hz), 131.5 (d, $J = 34.7$ Hz), 127.2 (d, $J = 4.5$ Hz), 126.5, 125.2, 122.5 (q, $J = 273.3$ Hz), 94.9, 65.0, 52.9, 45.9, 34.8, 31.7, 26.6, 25.9; HRMS (ESI+) *m/z* $[M + H]^+$ calcd for $C_{20}H_{24}F_3IN_3OS$, 538.0631; found, 538.0610.

3.2. General procedure for the preparation of compound A01−05, A07 via heck coupling reactions

To a solution of compound **C03** (1.0 equiv) in *N*,*N*-dimethylformamide (DMF) under a nitrogen atmosphere was added a series reagent containing propylene group (1.2 equiv; methyl acrylate, acrylamide, *N*-methacrylamide, *N*,*N*-dimethyl acrylamide, acrylonitrile, 2-vinylpyridine), triethylamine (2.0 equiv), palladium acetate (0.5 equiv). The reaction mixture was heated to 85 °C for 3−6 h. After being cooled to room temperature, the solvent was evaporated under reduced pressure and diluted with ethyl acetate (20 mL) and washed with 10 mL H_2O. The solvents were removed. The products were purified using flash chromatography using mixtures of EtOAc and PET as eluent. The isolated yields were 56−92%.

3.2.1. Methyl (E)-3-(2-(4-(cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H- benzo[e][1,3]thiazin-8-yl)acrylate (**A01**)

The title compound was prepared from **C03** and methyl acrylate using the general procedure for Heck reaction (yield 92%) as a light white solid: $R_f = 0.24$, EtOAc-PET (1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.75 (s, 1H), 7.91, 7.89 (d, $J = 16.0$ Hz, 1H), 6.54 (d, $J = 16.0$ Hz, 1H), 4.12 (br, 2H), 3.86 (s, 3H), 3.63 (br, 2H), 2.56 (s, 4H), 2.22 (d, $J = 4$ Hz, 2H), 1.80−1.67 (m, 6H), 1.51 (s, 1H), 1.32−1.28 (m, 2H), 0.93−0.87 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 167.8, 166.1, 160.2, 137.1, 135.9, 132.5, 130.0 (q, J = 33.2 Hz), 128.5 (d, J = 3.0 Hz), 126.3 (d, $J = 3.0$ Hz), 124.3, 123.9, 123.2 (q, $J = 273.3$ Hz), 65.1, 52.9, 52.2, 46.1, 34.9, 31.7, 26.6, 26.0; HRMS (ESI+) *m/z* $[M+H]^+$ calcd for $C_{24}H_{29}F_3N_3O_3S$, 496.1876; found 496.1893.

3.2.2. (E)-3-(2-(4-(Cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-8-yl)acrylamide (**A02**)

The title compound was prepared from **C03** and acrylamide using the general procedure for Heck reaction (yield 56%) as a brown solid: $R_f = 0.25$, EtOAc-PET (1:1); ^{1}H NMR (400 MHz, CD_3OD) δ 8.59 (s, 1H), 8.14 (s, 1H), 7.83 (d, J = 15.2 Hz, 1H), 6.83 (d, $J = 15.2$ Hz, 1H), 4.88 (s, 2H), 4.03 (br, 4H), 2.72 (br, 4H), 2.36 (s, 2H), 1.85−1.63 (m, 6H), 1.53 (br, 1H), 1.29−1.17 (m, 2H), 0.97−0.88 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.1, 164.9, 159.5, 135.1, 134.1, 131.8, 128.8 (q, $J = 33.5$ Hz), 127.1 (d, $J = 3.3$ Hz), 125.0 (d, $J = 2.9$ Hz),

124.6, 123.2, 122.3 (q, J = 271.3 Hz), 64.1, 52.0, 33.9, 30.7, 28.7, 25.7, 25.0; HRMS (ESI+) m/z $[M+H]^+$ calcd for $C_{23}H_{28}F_3N_4O_2S$, 481.1880; found, 481.1863.

3.2.3. (E)-3-(2-(4-(Cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-8-yl)-N-methylacrylamide (**A03**)

The title compound was prepared from **C03** and *N*-methyl acrylamide using the general procedure for Heck reaction (yield 56%) as a brown solid: R_f = 0.23, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.66 (s, 1H), 7.83 (d, J = 14.8, 1H), 7.82 (s, 1H), 6.56 (d, J = 15.2 Hz, 1H), 6.45 (d, J = 3.2 Hz, 1H), 4.08 (br, 2H), 3.76 (br, 2H), 2.98 (d, J = 4.4 Hz, 3H), 2.49 (s, 4H), 2.16 (d, J = 6.0 Hz, 2H), 1.78−1.69 (m, 6H), 1.48 (br, 1H), 1.31−1.19 (m, 2H), 0.91−0.85 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.1, 164.9, 160.6, 136.0, 133.7, 133.2, 129.7 (q, J = 28.2 Hz), 129.0 (q, J = 277.4 Hz), 127.9 (d, J = 3.6 Hz), 126., 125.9 (d, J = 2.7 Hz), 124.2, 65.1, 53.1, 53.0, 34.9, 31.7, 29.7, 26.7, 26.0; HRMS (ESI+) m/z $[M+H]^+$ calcd for $C_{24}H_{30}F_3N_4O_2S$, 495.2036; found: 495.2000.

3.2.4. (E)-3-(2-(4-(Cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-8-yl)-N,N-dimethylacrylamide (**A04**)

The title compound was prepared from **C03** (20 mg, 0.04 mmol) and *N,N*-dimethyl acrylamide using the general procedure for Heck reaction as a brown solid (yield 59%): R_f = 0.5, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.74 (s, 1H), 7.91 (d, J = 16.0 Hz, 1H), 7.89 (s, 1H), 6.98 (d, J = 16.0 Hz, 1H), 4.13 (br, 2H), 3.81 (br, 2H), 3.23 (s, 3H), 3.12 (s, 3H), 2.54 (br, 4H), 2.21 (br, 2H), 1.81−1.71 (m, 6H), 1.52 (br, 1H), 1.33−1.25 (m, 2H), 0.94−0.88 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.1, 165.0, 160.7, 135.9, 135.2, 133.6, 129.8 (d, J = 34.7 Hz), 127.9 (d, J = 3.0 Hz), 125.9 (d, J = 3.0 Hz), 124.2, 123.5, 123.3 (q, J = 273.3 Hz), 65.1, 53.0, 46.4, 37.5, 36.1, 34.9, 31.7, 26.6, 26.0; HRMS (ESI+) m/z $[M + H]^+$ calcd for$C_{25}H_{32}F_3N_4O_2S$, 509.2193; found 509.2207.

3.2.5. (E)-3-(2-(4-(Cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-8-yl)acrylonitrile (**A05**)

The title compound was prepared from **C03** and acrylonitrile using the general procedure for Heck reaction as a yellow solid (yield 76%): R_f = 0.4, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (s, 1H), 7.85 (s, 1H), 7.64 (d, J = 16.0 Hz, 1H), 6.05 (d, J = 16 Hz, 1H), 4.12 (br, 2H), 3.80 (br, 2H), 2.54 (br, 4H), 2.19 (s, 2H), 1.80−1.70 (m, 6H), 1.50 (br, 1H), 1.32−1.15 (m, 2H), 0.90−0.84 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 167.3, 159.5, 143.1, 135.7, 131.5, 130.2 (q, J = 34.7 Hz), 129.3 (d, J = 3.0 Hz), 125.8 (d, J = 1.5 Hz), 124.5, 123.0 (q, J = 273.3 Hz), 116.5, 103.0, 65.1, 53.0, 46.3, 34.9, 31.7, 26.7, 26.0; HRMS (ESI+) m/z $[M+H]^+$ calcd for $C_{23}H_{26}F_3N_4OS$, 463.1774; found, 463.1783.

3.2.6. Methyl3-(2-(4-(cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-8-yl)propiolate (**A06**)

To a stirred solution of compound **C03** (20 mg, 0.04 mmol) in tetrahydrofuran (THF, 5 mL) was added methyl propiolate (5 mg, 0.05 mmol, 1.2 equiv), potassium carbonate (10 mg, 0.08 mmol, 2.0 equiv), $Pd(PPh_3)_2Cl_2$ (2 mg, 0.2 equiv) and CuI (1 mg, 0.2 equiv.) and the resulting mixture was stirred for 4 h at room temperature. The reaction mixture was then diluted with water (10 mL) and extracted with DCM (10 mL × 3). The organic extracts were combined and dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The crude residue was purified by flash column chromatography on silica gel to afford compound **A06** (15 mg, yield 82%) as a brown solid: R_f = 0.23, EtOAc: PET = 1:5.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (s, 1H), 7.99 (s, 1H), 4.15 (br, 2H), 3.90 (s, 3H), 3.78 (br, 2H), 2.57 (br, 4H), 2.22 (s, 2H), 1.80−1.71 (m, 6H), 1.51 (br, 1H), 1.24−1.15 (m, 2H), 0.90−0.87 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 167.3, 160.6, 153.3, 140.5, 133.1 (d, J = 2.8 Hz), 130.1 (q, J = 34.4 Hz), 129.3 (d, J = 3.3 Hz), 124.2, 122.8 (q, J = 273.0 Hz), 117.8, 88.8, 78.9, 65.1, 53.3, 53.0, 46.3, 35.0, 31.7, 26.7, 26.0; HRMS (ESI+) m/z $[M+Na]^+$ calcd for $C_{24}H_{26}F_3N_3O_3SNa$, 516.1539; found, 516.1521.

3.2.7. (E)-2-(4-(Cyclohexylmethyl)piperazin-1-yl)-8-(2-(pyridin-2-yl)vinyl)-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-4-one (**A07**)

The title compound was prepared from **C03** and 2-vinylpyridine using the general procedure for Heck reaction as a brown solid (yield 57%): R_f = 0.35, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (s, 1H), 8.68 (d, J = 4.0 Hz, 1H), 8.01 (s, 1H), 7.90 (d, J = 16.0 Hz, 1H), 7.74 (t, J = 8.0 Hz, 1H), 7.40 (d, J = 4.0 Hz, 1H), 7.26 (s, 1H), 7.21 (d, J = 12.0 Hz, 1H), 4.07 (br, 2H), 3.83 (br, 2H), 2.51 (s, 4H), 2.17 (d, J = 8.0 Hz, 2H), 1.79−1.70 (m, 6H), 1.49 (br, 1H), 1.33−1.25 (m, 2H), 0.92−0.87 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.4, 161.0, 153.8, 149.9, 136.9, 135.1, 133.9, 129.8 (q, J = 33.2 Hz), 126.7, 125.5 (d, J = 3.0 Hz), 125.2, 124.0, 123.7, 123.5 (q, J = 273.3 Hz), 123.4, 65.0, 52.9, 45.8, 34.8, 31.7, 26.6, 25.9; HRMS (ESI+) m/z $[M+H]^+$ calcd for $C_{27}H_{30}F_3N_4OS$, 515.2087; found 515.2078.

3.3. General procedure for the synthesis of compound **C01**, **C02**

A mixture of *tert*-butyl nitrite (1.25 equiv), copper (I) chloride or copper (II) bromide (1.25 equiv), in CH_3CN was heated to 60 °C, then treated with intermediate **2** (1.0 equiv) over 10 min, stirring is continued for 2 h. The mixture was cooled to room temperature, and diluted with ethyl acetate. The organic layer was washed with 10% hydrochloric acid and filtered, concentrated. The residue was applied to column chromatography to afford the title compound **C01**and **C02.**

3.3.1. 8-Chloro-2-(4-(cyclohexylmethyl)piperazin-1-yl)-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-4-one (**C01**)

The title compound was prepared from intermediate **2** (100 mg, 0.24 mmol), copper (I) chloride (280 mg, 0.48 mmol, 2.0 eq) and *tert*-butyl nitrite (48 mg, 0.47 mmol, 2.0 eq.) using the above procedure as a white solid (58 mg, yield 54%): R_f = 0.68, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (s, 1H), 7.97 (s, 1H), 4.13 (br, 2H), 3.81 (br, 2H), 2.54 (br, 4H), 2.20 (s, 2H), 1.81−1.71 (m, 6H), 1.40 (br, 1H), 1.25−1.15 (m, 2H), 0.90−0.88 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.3, 161.2, 137.7, 131.9 (d, J = 1.5 Hz), 130.9 (d, J = 36.2 Hz), 126.5 (d, J = 3.0 Hz), 126.0, 122.7 (q, J = 273.3 Hz), 120.4, 65.0, 52.8, 46.0, 34.7, 31.7, 26.5, 25.9; HRMS (ESI+) m/z $[M+H]^+$ calcd for $C_{20}H_{24}ClF_3N_3OS$, 446.1275; found, 446.1294.

3.3.2. 8-Bromo-2-(4-(cyclohexylmethyl)piperazin-1-yl)-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-4-one (**C02**)

The title compound **C02** was prepared from intermediate **2** (50 mg, 0.12 mmol), copper (II) bromide (34 mg, 0.24 mmol, 2.0 equiv) and *tert*-butyl nitrite (15 mg, 0.14 mmol, 1.2 equiv) using the above procedure. The product was obtained as a white solid (32 mg, yield 52%): R_f = 0.48, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.72 (s, 1H), 7.97 (s, 1H), 4.15 (br, 2H), 3.85 (br, 2H), 2.59 (s, 4H), 2.24 (s, 2H), 1.77−1.71 (m, 6H), 1.53 (br, 1H), 1.41−1.25 (m, 2H), 0.88 (br, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.3, 161.1, 137.9, 131.8, (d, J = 3.0 Hz), 130.8 (d, J = 33.2 Hz), 126.4 (d, J = 3.0 Hz), 126.1, 126.0 (d, J = 273.3Hz), 120.3, 65.1, 53.0, 46.3, 34.9, 31.7, 26.7, 26.0; HRMS (ESI+) m/z $[M+Na]^+$ calcd for $C_{20}H_{24}BrF_3N_3OSNa$, 512.0590; found, 512.0755.

3.3.3. 2- (4-(Cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazine-8-carbonitrile (**C04**)

To a solution of compound **C03** (20 mg, 37.2 μmol) in DMF

(10.0 mL) under nitrogen atmosphere was added zinc (II) cyanide (3 mg, 0.02 mmol) and tetrakis (triphenylphosphine)palladium (0) (2 mg, 10%). The reaction mixture was stirred at 90 °C for 4 h and was then diluted with toluene (10 mL) and the phases were separated. The aqueous phase was extracted twice with toluene (10 mL × 2). The combined organic phase was washed with brine (10 mL) and saturated aqueous ammonium hydroxide, dried over Na_2SO_4 and concentrated. The crude residue was purified by flash column chromatography on silica gel to afford compound **C04** (14 mg, yield 86%) as a white solid: $R_f = 0.26$, EtOAc: PET (1:5); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (s, 1H), 8.08 (s, 1H), 4.15 (br, 2H), 3.78 (br, 2H), 2.54 (s, 4H), 2.19 (s, 2H), 1.80−1.71 (m, 6H), 1.67 (br, 1H), 1.28−1.18 (m, 2H), 0.90−0.87 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.3, 159.2, 140.5, 132.7 (d, $J = 2.7$ Hz), 131.5 (d, $J = 3.0$ Hz), 130.7 (q, $J = 34.9$ Hz), 124.6, 122.3 (q, $J = 273.8$ Hz), 113.9, 110.9, 65.0, 53.1, 35.0, 31.7, 29.7, 26.7, 26.0; HRMS (CI+) *m/z* $[M+H]^+$ calcd for $C_{21}H_{24}F_3N_4OS$, 437.1617; found, 437.1614.

3.4. General procedure for the synthesis of compound ***D01***, ***D02***, ***D03***

To a solution of compound A01, or A02 or A05 in methanol was added 20% Pd/C. The mixture was stirred under hydrogen at room temperature. After completion, the mixture was filtered through Celite and the filtrate was concentrated. The residue was applied to column chromatography for purification to afford the title compound **D01**, **D02**, **D03**.

3.4.1. Methyl-3-(2-(4-(cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-8-yl)propanoate (***D01***)

The title compound was prepared from **A01** (20 mg, 0.04 mmol) and Pd/C (4 mg, 20%) using the general procedure for reduction reaction (18 mg, yield 90%) as a cyan-blue solid: $R_f = 0.34$, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (s, 1H), 7.61 (s, 1H), 4.07 (br, 2H), 3.79 (br, 2H), 3.70 (s, 3H), 3.11 (t, $J = 8.0$ Hz, 2H), 2.73 (t, $J = 8.0$ Hz, 2H), 2.51 (s, 4H), 2.18 (d, $J = 8.0$ Hz, 2H), 1.79−1.70 (m, 6H), 1.49 (s, 1H), 1.27−1.22 (m, 2H), 0.89−0.86 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.1, 168.5, 160.7, 137.1, 135.3, 129.5 (q, $J = 33.2$ Hz), 128.1 (d, $J = 3.0$ Hz), 125.9 (d, $J = 3.0$ Hz), 123.8, 123.5 (q, $J = 273.3$ Hz), 65.1, 53.0, 51.9, 46.2, 34.9, 33.0, 31.7, 27.7, 26.7, 26.0; HRMS (ESI+) *m/z* $[M+H]^+$ calcd for$C_{24}H_{31}F_3N_3O_3S$, 498.2033; found, 498.2009.

3.4.2. 3-(2-(4-(cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-8-yl)propanenitrile (***D02***)

The title compound was prepared from **A05** (20 mg, 0.04 mmol) and Pd/C (4 mg, 20%) using the general procedure for reduction reaction (18 mg, yield 95%) as a cyan-blue solid: $R_f = 0.36$, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (s, 1H), 7.65 (s, 1H), 4.10 (br, 2H), 3.79 (br, 2H), 3.17 (t, $J = 8.0$ Hz, 2H), 2.76 (t, $J = 8.0$ Hz, 2H), 2.52 (s, 4H), 2.18 (d, $J = 8.0$ Hz, 2H), 1.79−1.67 (m, 6H), 1.49 (br, 1H), 1.28−1.20 (m, 2H), 0.95−0.86 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.1, 159.9, 135.3, 134.7, 129.8 (q, $J = 33.2$ Hz), 128.5, 126.7, 124.1, 121.3 (q, $J = 271.8$ Hz), 117.8, 65.1, 53.0, 46.4, 35.0, 31.7, 28.4, 26.7, 26.0, 17.1; HRMS (ESI+) *m/z* $[M+H]^+$ calcd for$C_{23}H_{28}F_3N_4OS$, 465.1930; found, 465.1912.

3.4.3. 3-(2-(4-(Cyclohexylmethyl)piperazin-1-yl)-4-oxo-6-(trifluoromethyl)-4H-benzo[e][1,3]thiazin-8-yl)propanamide (***D03***)

The title compound was prepared from **A02** (20 mg, 0.04 mmol) and Pd/C (4 mg, 20%) using the general procedure for reduction reaction (19 mg, yield 98%) as a white solid: $R_f = 0.28$, EtOAc-PET 1:1; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (s, 1H), 7.64 (s, 1H), 5.65 (s, 1H), 5.54 (s, 1H), 4.24 (br, 2H), 4.03 (br, 2H), 3.14 (s, 2H), 2.63 (s, 6H), 2.29 (s, 2H), 1.83−1.72 (m, 6H), 1.60 (s, 1H), 1.28−1.21 (m, 2H), 0.94−0.87 (br, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 168.6, 160.9, 137.5, 135.3, 128.1, 125.9, 123.8, 65.1, 52.9, 46.1, 34.4, 31.7, 29.7, 27.7, 26.6, 26.0; HRMS (ESI+) *m/z* $[M+H]^+$ calcd for $C_{23}H_30F_3N_4O_2S$, 483.2036; found, 483.2062.

3.5. Preparation of intermediate ***5***

Oxalyl chloride (165 mg, 1.3 mmol, 2.5 equiv) and catalytic amount of DMF were added successively to the suspension of 2,6-dichloronicotinic acid (100 mg, 0.52 mmol) in DCM (10 mL). The reaction was stirred at room temperature for 1 h and evaporated in vacuo. The residue was dissolved in 20 mL DCM and added to ammonium thiocyanate (59 mg, 0.78 mmol, 1.5 equiv) dropwise under stirring. A catalytic amount of PEG-400 was added to this suspension, it was then stirred at room temperature for 20 min. After consumption of the starting material, 1-(cyclohexylmethyl) piperazine (95 mg, 0.52 mmol) was added and stirred for 1 h at room temperature. The reaction mixture was diluted with water (30 mL) and extracted with DCM (20 mL × 3). The combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc-PET (1:3) to afford intermediate **5** as a colorless liquid (142 mg, yield 64%): $R_f = 0.27$, EtOAc: PET (1:3). MS(+ESI) *m/z* calcd for $C_{18}H_{25}Cl_2N_4OS$ $[M+H]^+ = 415.10$, found, 414.7.

3.6. Preparation of intermediate ***6***

To a solution of intermediate **5** (20 mg, 0.04 mmol) in 10 mL toluene was added triethylamine (67 mg, 0.66 mmol, 2.0 equiv). The reaction mixture was stirred at 100 °C for 1 h and then diluted with 10 mL H_2O and the phases were separated. The aqueous phase was extracted twice with toluene (10 mL × 2). The combined organic phase was washed with brine and saturated aqueous ammonium hydroxide, dried over sodium sulfate and concentrated. The crude residue was purified by flash column chromatography on silica gel to afford intermediate **6** as a white solid (12 mg, yield 92%). EtOAc-PET (1:3, $R_f = 0.24$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (d, $J = 8.2$ Hz, 1H), 7.38 (d, $J = 8.2$ Hz, 1H), 4.12 (s, 2H), 3.69 (s, 2H), 2.51 (s, 4H), 2.17 (d, $J = 6.8$ Hz, 2H), 1.76 (d, $J = 14.8$ Hz, 4H), 1.69 (s, 2H), 1.48 (s, 1H), 1.28−1.21 (m, 2H), 0.93−0.85 (m, 2H).

3.6.1. 2-(4-(Cyclohexylmethyl)piperazin-1-yl)-4-oxo-4H-pyrido[3,2-e][1,3]thiazine-7-carbonitrile (***B01***)

To a 20 mL microwave vial containing a mixture of intermediate **6** (30 mg, 0.08 mmol), zinc cyanide (11 mg, 0.1 mmol, 1.2 equiv), zinc powder (4 mg, 0.02 mmol, 0.2 equiv), and DPPF (9 mg, 0.02 mmol, 0.2 equiv) was added DMSO (6 mL). The suspension was stirred with degassing under nitrogen for 5 min. Next, tris(dibenzylideneacetone)dipalladium (0) (7 mg, 0.01 mmol, 0.1 equiv) was added. The reaction mixture was sealed and stirred with heating at 100 °C (heat block) for 20 min. Then it was allowed to cool to RT, diluted with 20 mL of water, and adjusted to pH~8 with sat $NaHCO_3$, and extracted with DCM (10 mL × 3). The organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel to afford compound **B01** as a white solid (20 mg, yield 65%): EtOAc-PET (1:3, $R_f = 0.23$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.75 (d, $J = 8.0$ Hz, 1H), 7.75 (d, $J = 8.0$ Hz, 1H), 4.15 (br, 2H), 3.72 (br, 2H), 2.53 (s, 4H), 2.18 (s, 2H), 1.79−1.70 (m, 6H), 1.49 (br, 1H), 1.27−1.20 (m, 2H), 0.92−0.87 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 167.6, 161.9, 156.5, 139.1, 135.7, 127.4, 122.6, 115.8, 65.0, 53.0, 34.9, 31.7, 29.7, 26.7, 26.0; HRMS (CI+) *m/z* $[M+H]^+$ calcd for $C_{19}H_{24}N_5OS$, 370.1696; found, 370.1700.

3.6.2. 7-Chloro-2-(4-(cyclohexylmethyl)piperazin-1-yl)-4H-pyrimido[5,4-e][1,3]thiazin-4-one (**B02**)

To a solution of 2,4-dichloropyrimidine-5-carbonyl chloride (100 mg, 0.47 mmol) in DCM (10 mL) added ammonium thiocyanate (54 mg, 0.71 mmol, 1.5 equiv) dropwise under stirring. A drop of PEG-400 was added to the suspension, it was then stirred at room temperature for 20 min. After consumption of the starting material, 1-(cyclohexylmethyl)piperazine (86 mg, 0.47 mmol) was added. It was then stirred for 1 h at room temperature. The reaction mixture was then diluted with water (30 mL) and extracted with DCM (20 mL × 3). The organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel to afford compound **B02** as a white solid (159 mg, yield 89%): EtOAc-PET (1:3, $R_f = 0.27$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.36 (s, 1H), 4.17 (br, 2H), 3.74 (br, 2H), 2.57 (s, 4H), 2.21 (s, 2H), 1.81–1.73 (m, 6H), 1.51 (br, 1H), 1.35–1.23 (m, 2H), 0.92–0.88 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 167.3, 166.6, 162.3, 161.0, 160.0, 115.9, 65.0, 53.0, 52.8, 47.0, 46.6, 35.0, 31.6, 26.7, 26.0; HRMS (CI+) *m/z* $[M+H]^+$ calcd for $C_{17}H_{23}ClN_5OS$, 380.1306; found, 380.1317.

3.6.3. 2-(4-(Cyclohexylmethyl)piperazin-1-yl)-4-oxo-4H-pyrimido[5,4-e][1,3]thiazine-7-carbonitrile(**B03**)

To a solution of compound **B02** (50 mg, 0.13 mmol) in DMSO (5 mL) was added zinc cyanide (19 mg, 0.16 mmol, 1.2 equiv) and DPPF (14.59 mg, 26.32 μmol, 0.2 equiv). The suspension was stirred with degassing under nitrogen for 5 min. Next, tris(dibenzylideneacetone)dipalladium (0) (15 mg, 0.03 mmol, 0.2 equiv) was added. The reaction mixture was sealed and stirred with heating at 80 °C for 2 h. Then it was allowed to cool to RT, diluted with 20 mL of water, and adjusted to pH~8 with sat $NaHCO_3$, extracted with DCM (10 mL × 3). The organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel to afford compound **B03** (26 mg, yield 53%) as a white solid: EtOAc-PET (1:3, $R_f = 0.28$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.48 (s, 1H), 4.13 (s, 2H), 3.72 (s, 2H), 2.56 (s, 2H), 2.50 (s, 2H), 2.17 (d, J = 6.8 Hz, 2H), 1.77–1.69 (m, 6H), 1.47 (br, 1H), 1.28–1.21 (m, 2H), 0.90–0.85 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 166.4, 165.8, 159.8, 159.1, 144.9, 118.3, 114.6, 64.9, 53.0, 52.8, 47.3, 46.8, 35.0, 31.6, 26.7, 26.0; HRMS (CI+) *m/z* $[M+H]^+$ calcd for $C_{18}H_{23}N_6OS$, 371.1649; found, 371.1657.

3.7. Reactivity of compounds with methyl thioglycolate in PBS buffer

To assess the compound reactivity with methyl thioglycolate, the prepared compounds (10 mM) and methyl thioglycolate (50 mM) was incubated at 37 °C in 50 mM PBS buffer (pH 7.4) in a total volume of 160 μL. The reaction was monitored by TLC and analyzed by injection of aliquots to LC–MS/MS at predetermined intervals within a period of 48 h. Control reaction was run in the absence of methyl thioglycolate. The formation of methyl thioglycolate adduct was determined by detection of the corresponding MS adduct peak.

3.7.1. $T_{1/2}$ Determination

The above reaction was monitored by HPLC. The disappearance of the parent compounds was monitored as % remaining relative to time zero, and the data were fitted to first-order kinetics by plotting the natural log of % remaining as a function of time. The pseudo-first-order rate constant (k), which is the negative slope of the linear fitting, was used to calculate the reaction half-life ($t_{1/2} = 0.693/k$).

3.7.2. MIC determination

M. tuberculosis H37Rv (ACTT 25618) strains were subcultured on Lowenstein–Jensen medium at 37 °C for 28 days. Bacterial suspension was adjusted to a turbidity equivalent to 1.0 McFarland standard, then further diluted 1:20 in Middle brook 7H9 broth supplemented with 10% (vol/vol) OADC enrichment and 0.2% (vol/vol) glycerol. MICs against replicating *M.* tuberculosis were determined by the microplate alamar blue assay (MABA) [19]. PBTZ 169 was used as positive control. Compound stock solutions (1.0 mg/mL) in DMSO were prepared and serially diluted in two-fold. For the most active compounds, the stock solution concentration was 3.2 μg/mL and the final testing concentration range was 2 to 0.002 μg/mL, respectively.

100 μL of bacterial culture suspension was added to the wells of the 96-well plate containing the corresponding drugs, and the plates were incubated at 37 °C. On day 7, 50 μL of 5% Tween 80 and 20 μL of alamar blue were added to all wells. After incubation for 24 h, the colors of all wells were recorded. A color change from blue to pink indicates bacterial growth. MIC was defined as the lowest concentration of the drug that showed no color change, which was the lowest concentration of drug capable of inhibiting the visible growth of tested isolates.

3.7.3. IC_{50} determination

M. tuberculosis DprE1 was expressed and purified in *E. coli* cells, as previously reported [20]. Enzyme activity was determined using the Amplex Red/peroxidase coupled assay was performed according to published procedure [21]. Briefly, DprE1 (0.5 μM) was incubated at 30 °C in 20 mM glycylglycine pH 8.5, containing, 0.050 mM Amplex Red, and 0.35 μM horseradish peroxidase. The reaction was initiated by adding 500 μM FPR, and the formation of resorufin was followed at 572 nm ($\varepsilon = 54{,}000\ M^{-1}\ cm^{-1}$). Initial inhibition assays were carried out in the presence of 20 μM of each compound (dissolved in DMSO). DMSO was used as negative control, PBTZ169 as positive control. Compounds with more than 70% inhibitory activity at 20 μM were further analyzed for IC_{50} calculated according to equation:

$$A_{[I]} = A_{[0]} \times \left(1 - \frac{[I]}{[I] + IC_{50}}\right)$$

where $A_{[I]}$ is the activity of the enzyme at inhibitor concentration [I] and $A_{[0]}$ is the activity of the enzyme without inhibitor. Otherwise, the activity was recorded as "a", as indicated in Table 1. All results are mean SD of three replicates.

3.7.4. Covalent modification

DprE1 (3 mg/mL, 100 μl) was incubated with 100 μM **A01** or **A02** at 30 °C for 30 min. The sample was dialyzed, concentrated to 20 μL, diluted 1:5 in methanol-water 1:1 containing 0.01% formic acid, and directly analyzed in mass spectrometry, using an Ion Trap (LCQ Fleet™) mass spectrometer with electrospray ionization (ESI) ion source controlled by Xcalibur software 2.2. Mass spectra, recorded for 2 min, were generated in positive ion mode under constant instrumental conditions: source voltage 5.0 kV, capillary voltage 46 V, sheath gas flow 20 (arbitrary units), auxiliary gas flow 10 (arbitrary units), sweep gas flow 1 (arbitrary units), capillary temperature 210 °C, tube lens voltage 105 V. MS/MS spectra, obtained by CID studies in the ion trap, were performed with an isolation width of 3 Da m/z, the activation amplitude was 35% of ejection RF amplitude that corresponds to 1.58 V.

Spectra deconvolution was performed using UniDec 3.2.0 software [22].

To identify the site of attachment of the compound the DprE1 reacted with **A01** and **A02** was digested with trypsin and then

peptides analyzed. Briefly, DprE1 (10 mg/mL, 100 μl) was incubated with 300 μM **A01** or **A02** at 30 °C for 60 min. As control the protein was incubated with DMSO, then samples were then incubated with 2 μg trypsin (Trypsin Gold, Promega) at 37 °C overnight. Peptides analyses were carried out on an LC-MS (Thermo Finnigan, San Jose, CA, United States) system consisting of a thermostated column oven Surveyor autosampler controlled at 25 °C; a quaternary gradient Surveyor MS pump equipped with an UV/V is detector and an Ion Trap (LCQ Fleet™) mass spectrometer with electrospray ionization (ESI) ion source controlled by Xcalibur software 2.2. Analytes were separated by RP-HPLC on a Jupiter (Phenomenex, Torrance, CA, United States) C18 column (150 × 2 mm, 4 μm, 90 Å particle size) using a linear gradient (2–60% solvent B in 45 min; 60–98% B in 5 min and 98% B in 10 min) in which solvent A consisted of 0.1% aqueous formic acid and solvent B of acetonitrile containing 0.1% formic acid. Flow-rate was 0.2 mL/min. Mass spectra were generated by using the same conditions described above.

3.8. Metabolic labeling

M. tuberculosis H37Rv were grown in 7H9/ADC/Tween80 medium until OD = 1.04 (Experiment 1) or OD = 0.64 (Experiment 2). Metabolic labeling was performed in the final volume of the culture 100 μL at concentration of the radiolabel 1 μCi/mL [^{14}C]-acetate, (specific activity 106 mCi/mL, American Radiolabeled Chemicals, Inc.). Drug treatment, extraction of the lipids with organic solvents and their TLC analysis was performed as described [18].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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