Supporting Information

2-Mercapto-quinazolinones as inhibitors of NDH-2 and *Mycobacterium tuberculosis*: Structure-activity relationships, mechanism of action and ADME characterization.

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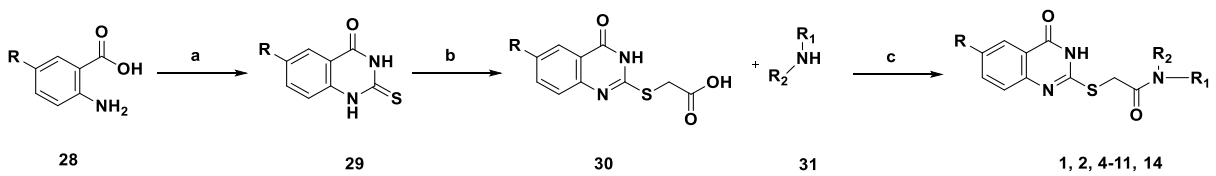
Supplemental Synthetic Schemes

Quinazolinone amides in **Table 2** were synthesized utilizing known procedures (**Scheme 1**). Commercially available anthranilic acids (**28**) were cyclized with thiourea, and the resulting 2-mercapto quinazoline-4-diones (**29**) or commercially available 2-mercapto-4(3H)-quinazolinone (CAS.No.13906-09-7) was reacted with 2-bromoaceticacid to form the 2-((4-oxo-3,4-dihydroquinazolin-2-yl)thio)acetic acids (**30**) or commercially available [(4-oxo-3,4-dihydroquinazolin-2-yl)sulfanyl]acetic acid from Vitas-M laboratory. Primary and secondary amines (**31**) were used in the couplings, with *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (*HATU*) or *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide.HCl (*EDC.HCl*), 1-Hydroxy-7-azabenzotriazole *(HOAT*) under basic condition as coupling agents to afford compounds **1, 2, 4-11, 14 and 26** on purification and characterization.

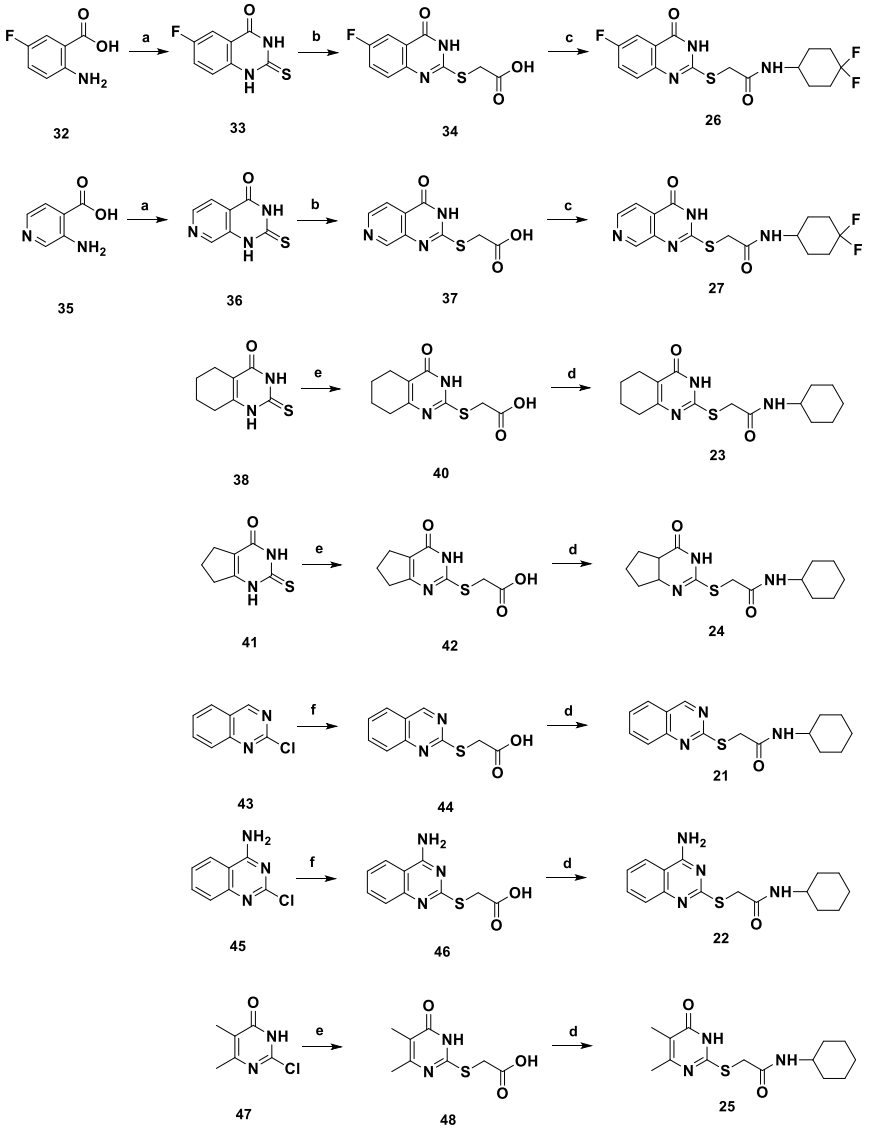
Scheme 1.



Reagents and conditions: General synthetic approach to quinazolinones **1, 2, 4-11 and 14**. (a) Neat thiourea at 180°C, 3h; (b) 2-Bromoacetic acid, triethylamine, DMF, 80°C, 12h; (c) Primary and or secondary amine **31**, EDC.HCl, HOAT, *N*,*N*-Diisopropylethylamine, DMF:ACN[1:1], room temperature, 12 h; or Primary and or secondary amine **31,** *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide **(***HATU*), *N*,*N*-Diisopropylethylamine, DCM, room temperature, 12 h; or cyclohexylamine**,** *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide **(***HATU*) *N*,*N*-Diisopropylethylamine, DCM, room temperature, 12 h.

Compounds involving quinzaolinone ring modifications were synthesized in accordance with the procedures in **Scheme 1**, starting with key building blocks outlined below (**Scheme 2**).

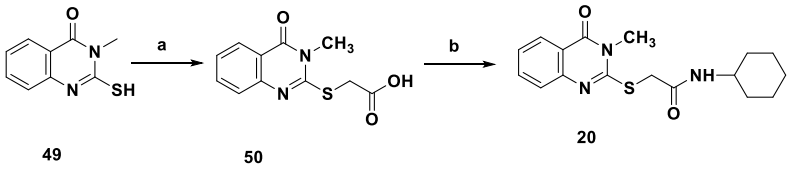
Scheme 2



Reagents and conditions: Synthetic Approach of Compound **26, 27, 23, 24, 21, 22,** and **25**; (a) Neat thiourea at 180°C, 3h; (b) 2-Bromoacetic acid **39**, triethylamine, DMF, 80°C, 12h; (c) 4,4-Difluorocyclohexan-1-amine , *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (*HATU*), *N*, *N*-diisopropylethylamine, DCM, room temperature, 12 h and (d) Cyclohexylamine, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (*HATU*), *N*, *N*-diisopropylethylamine, DCM, room temperature, 12 h; (e) Chloroacetic acid **39a**, DMF, triethylamine, r.t; (f) Sodium thioglycolic acid **39b**, DMF, triethylamine, 100°C

Introducing methyl (-CH_3) group in the C-3 position (**Scheme 3**) by commercial available starting material like 2-mercapto-3-methylquinazolin-4(3H)-one reacts (**49**) reacts with 2-bromoacetic acid in presence of basic condition to obtain the 2-((3-methyl-4-oxo-3,4-dihydroquinazolin-2-yl)thio)acetic acid (**50**), 87 % yield. Finally by amide coupling with cyclohexylamine & 2-((3-methyl-4-oxo-3,4-dihydroquinazolin-2-yl)thio)acetic acid (**50**) to gave the N-cyclohexyl-2-((3-methyl-4-oxo-3,4-dihydroquinazolin-2-yl)thio)acetamide (**20**).

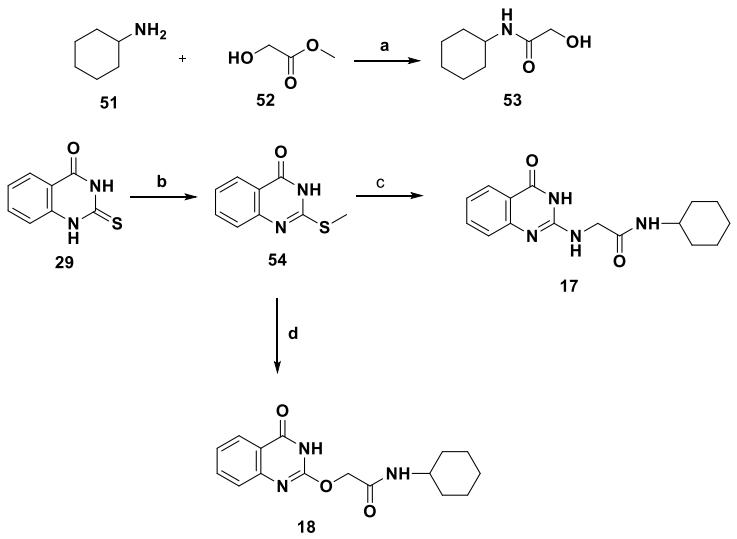
Scheme 3



Reagents and conditions: Synthetic Approach of Quinazoline **20,** (a) 2-Bromoacetic acid, triethylamine, DMF, 80°C, 12h; (b) Cyclohexylamine, *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide.HCl (EDC.HCl), HOAT, *N*,*N*-Diisopropylethylamine, DMF:ACN[1:1], RT, 12 h;

Synthesis of the *O*-linker analogue, was achieved by displacement of CH_3S-linker of 2-(methylthio)quinazolin-4(3H)-one with *N*-cyclohexylglycine (**53**), to afford *O*-Linker (**18**) in a low yield. Similarly, displacement of *CH_3S*-linker of 2-(methylthio)quinazolin-4(3H)-one with 2-amino-N-cyclohexylacetamide (**55**) affords *N*-linker (**17**) in moderate yield (**Scheme 4**).

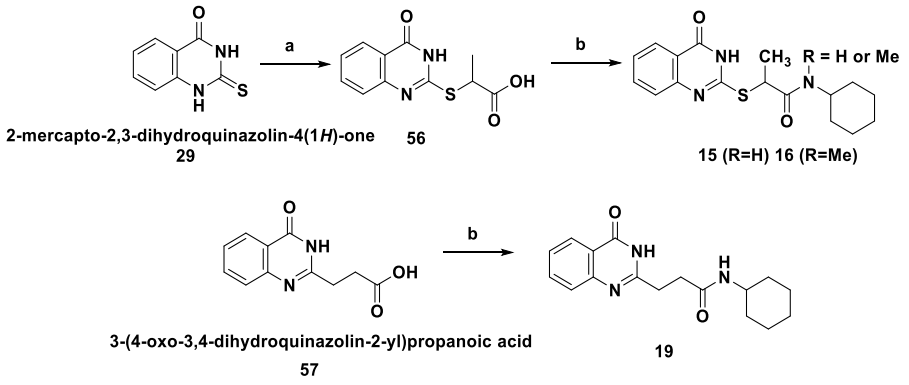
Scheme 4



Reagent and conditions: Synthesis of *N*-linker **17** and *O*-linker **18.** (a) neat 3h; (b) Iodomethane, 1% aqueous NaOH, methanol, 0°C, pH 1N HCl; (c) 2-Amino-*N*-cyclohexylacetamide **55**, ethyl acetate, microwave irradiation at 140°C (0-400 W at Ghz), 2h; (d) *N*-Cyclohexylglycine **53**, THF at reflux, 3h

Linker variation. Compound **15** and **16** can be synthesised from commercial available 2-mercapto quinazoline-4-dione by reaction with 2-bromopropanoic acid, amide coupling to the intermediate acid affords **15** and **16**. Replacement of *S*- to CH_2-linker was achieved using commercial available 3-(4-oxo-3,4-dihydroquinazolin-2-yl)propanoic acid (**57**) to undergo amide coupling with cyclohexylamine (**4**) to afford the final compound **19** (**Scheme 5**).

Scheme 5



Reagents and conditions: Synthetic of **15**, **16** and **19.** (a) 2-bromopropanoic acid, triethylamine, DMF, 75°C, 3h; (b) Cyclohexylamine,/ N-methylcyclohexanamine/ *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide.HCl (EDC.HCl), HOAT, *N*,*N*-Diisopropylethylamine, DMF:ACN[1:1], RT, 12 h

Met-ID studies using Mouse Liver Microsomes - Microsomal incubations were prepared by adding compound to 50 mM sodium phosphate buffer (pH 7.4) and 0.5mg/mL mouse liver microsomes to give a final incubated concentration of 5 µM. The incubations were initiated with the addition of NADPH (2 mM final concentration). Aliquots of 50µL were taken at 0, 3, 10, 60 and 120 min and mixed with 100µL of acetonitrile. 50µL of water was then added and the sample centrifuged at 4000rpm for 10min before removing the supernatent for analysis.

Met-ID studies using Human Liver Microsomes + GSH - Microsomal incubations were prepared by adding compound to 50 mM sodium phosphate buffer (pH 7.4) and 0.5mg/mL mouse liver microsomes to give a final incubated concentration of 5 µM.

A 1mL solution of NADPH regenerating system (RGS) was prepared by adding 7.8mL of Glucose-6-phosphate (27.65mM G6P), 1.7mL NADP (2.22mM), 6 units of G6P dehydrogenase (1.6µL) to 1 mL of a 2% (w/v) sodium bicarbonate solution.

A 50mM solution of GSH as prepared by dissolving 28mg of GSH in 1.8mL of 50 mM sodium phosphate buffer (pH 7.4). The final concentration of the GSH in the incubation mix was 5mM

The compound was added to the microsomal solution and pre-incubated at 37^{o}C for 5min before addition of the GSH and RGS solutions. The following control samples were also prepared:-

1. No GSH
2. No RGS
3. No compound

100µL of each incubation mix was added to 200µL of acetonitrile and stored at 4^{o}C. Samples were incubated at 37^{o}C for 90min and a second aliquot taken and added to acetonitrile. All samples were then centrifuged (4000rpm, 10min 4^{o}C) and 150µL of the supernatant added to 150µL water before analysis by UPLC-MS/MS

Analysis of Met-ID studies

All Samples were analysed on a Waters Xevo Q-TOF with Acquity UPLC and a Waters BEH C18 50x2.1mm 1.7µm particle size column. A flow rate of 0.5mL/min and a 7 min gradient using acidic eluents (A: H_2O +

0.1% formic acid, B: ACN + 0.1% formic acid) was used. Samples were analysed in positive ion with a capillary voltage of 2.25kV, source temperature of 120C and desolvation temperature of 500C. Cone voltage and collision energies were varied to give the maximum sensitivity and fragment coverage for each compound.

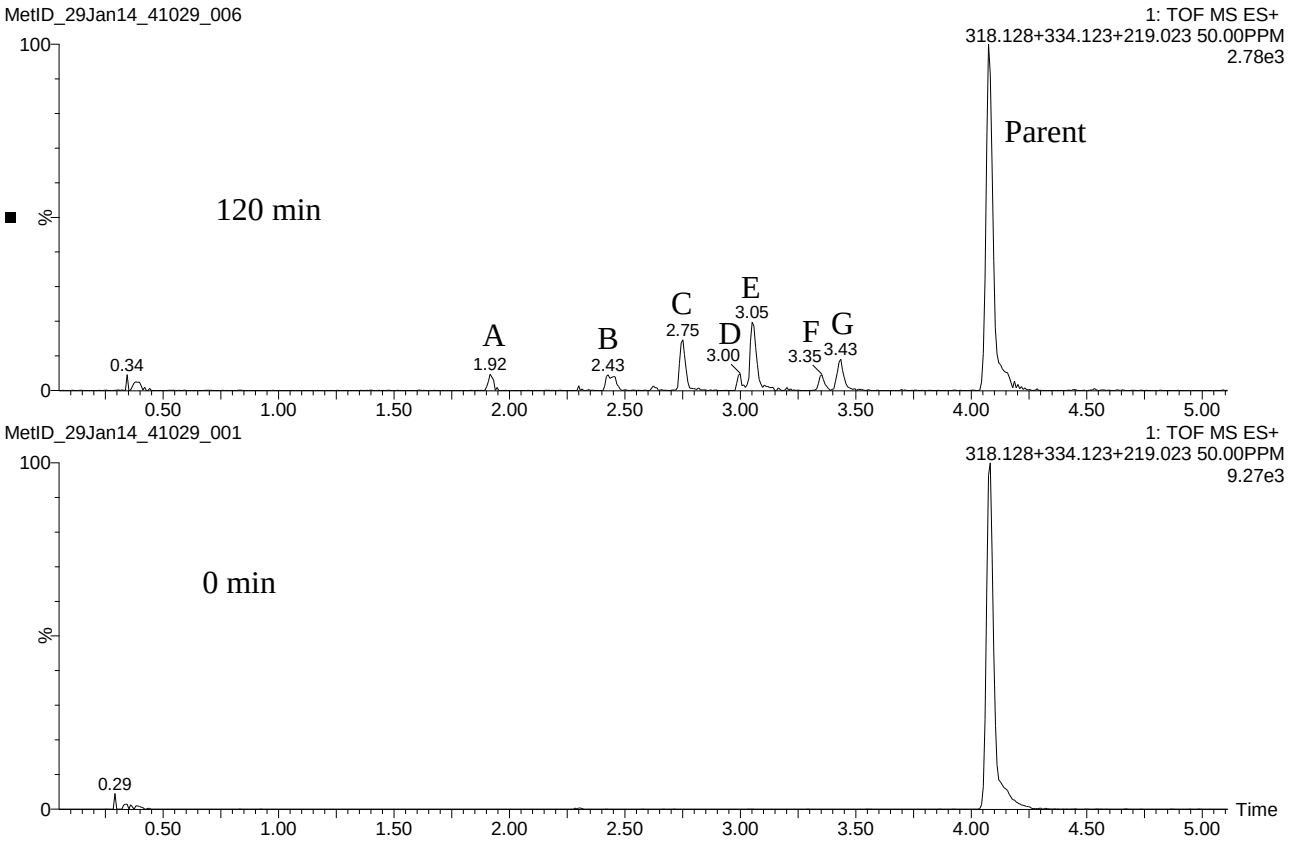
Whole-Genome Sequencing of Resistant Mutants

Nine isogenic mutants were selected for resistance to compound **1** at 10X MIC concentrations on 7H11/OADC agar plates. The mutants were selected from 3 different parental strains, 6 from *M. tuberculosis* H37Rv [1], 2 from *M. tuberculosis* H37RvMA (ATCC 27294)[2] and 1 from a drug-sensitive clinical isolate in the Beijing lineage. Resistance was confirmed by MIC determination in liquid media. DNA was extracted from isolated colonies using the CTAB-lysozyme protocol [3]. The samples were prepared using the standard Illumina whole-genome sample prepartion kit (Illumina, Inc.; San Diego, CA) and sequencing on an Illumina HiSeq 2500 sequencer, collecting paired-end reads with a read-length of 106 bp. The mean depth of coverage was 134.0 (range: 116-175). Reads were mapped onto the appropriate reference genome using BWA [4], and insertions and deletions (indels) were identified using local contig-building, as described in[2]. Polymorphisms were identified by aligning each genome to the sequence of the corresponding parental strain and tabulating single-nucleotide polymorphisms (SNPs) and indels, excluding those in heterogeneous sites (majority nucleotide <70%) and sites in low-coverage (<10x) or repetitive regions. Genetic differences shared among all the mutants derived from a given parent were also filtered out.

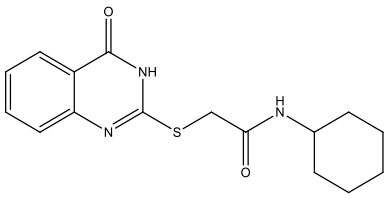
The polymorphisms identified among the resistant mutants are summarized in Table S1. Two of the mutants exhibited mutations in the promoter region of *ndhA* (Rv0392c), one of which was previously reported (-44 bp upstream of the translational start site), and a novel 7 bp deletion in the same region of the promoter (nucleotides -47 to -53). Three of the mutants exhibited loss-of-function mutations in Rv0678 (a regulator of MmpL5/S5), which likely confers resistance to 1 by acting as an eflux pump. Of the four remaining mutants, two had no polymorphisms that could be identified, and two had mutations whose potential role in resistance to 1 is unknown. These mutants all had unmutated parental sequences for the

2 NDH paralogs (Rv0392c and Rv1854c) and promoter regions. Furthermore, the genomes were checked for large-scale duplications, but they did not show evidence of duplications anywhere in the genome.

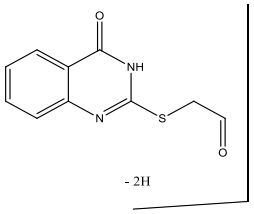
Supplementary Figures



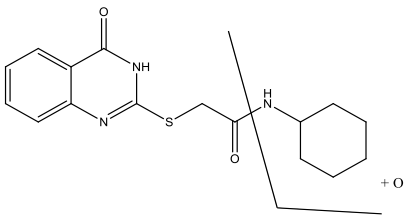
Proposed Structures



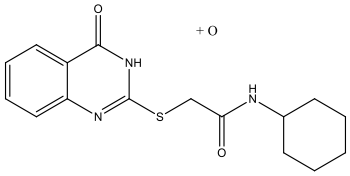
Parent



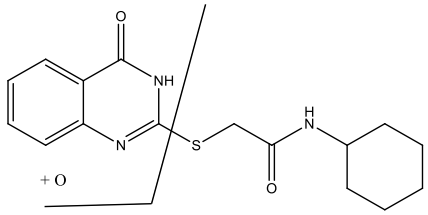
A – Cleave at amide bond



B, C, D and E – Oxidation

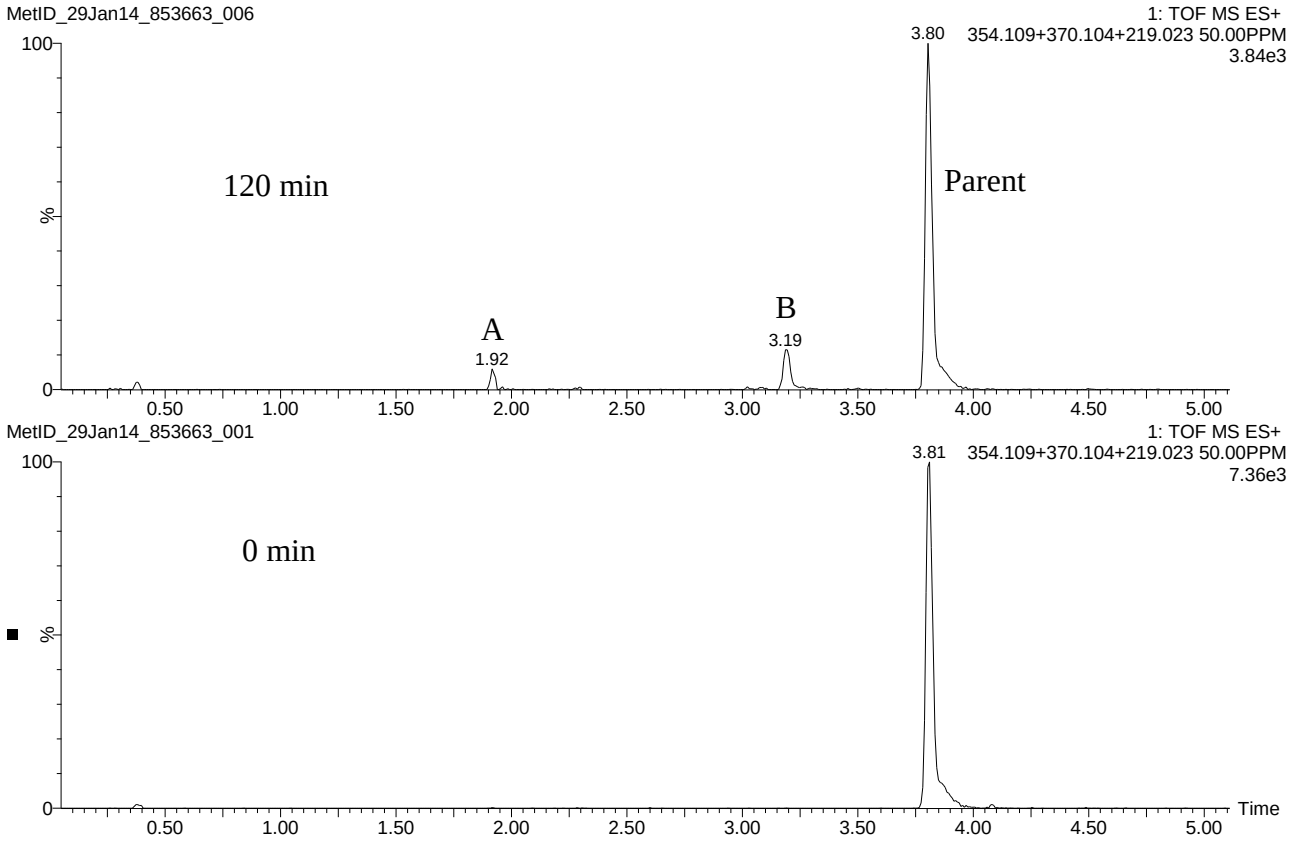


F – Oxidation

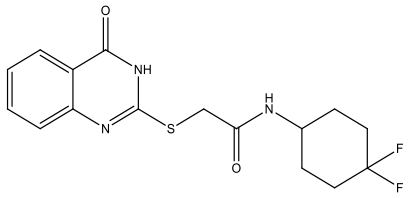


G - Oxidation

***Figure S1*. Met-ID studies on compound *1* during incubation with mouse liver microsomes.**



Proposed Structures



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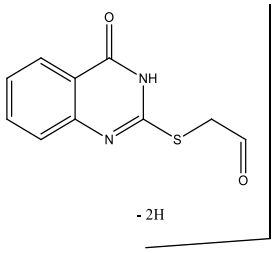
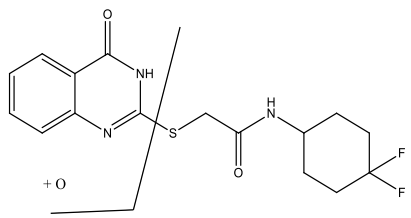
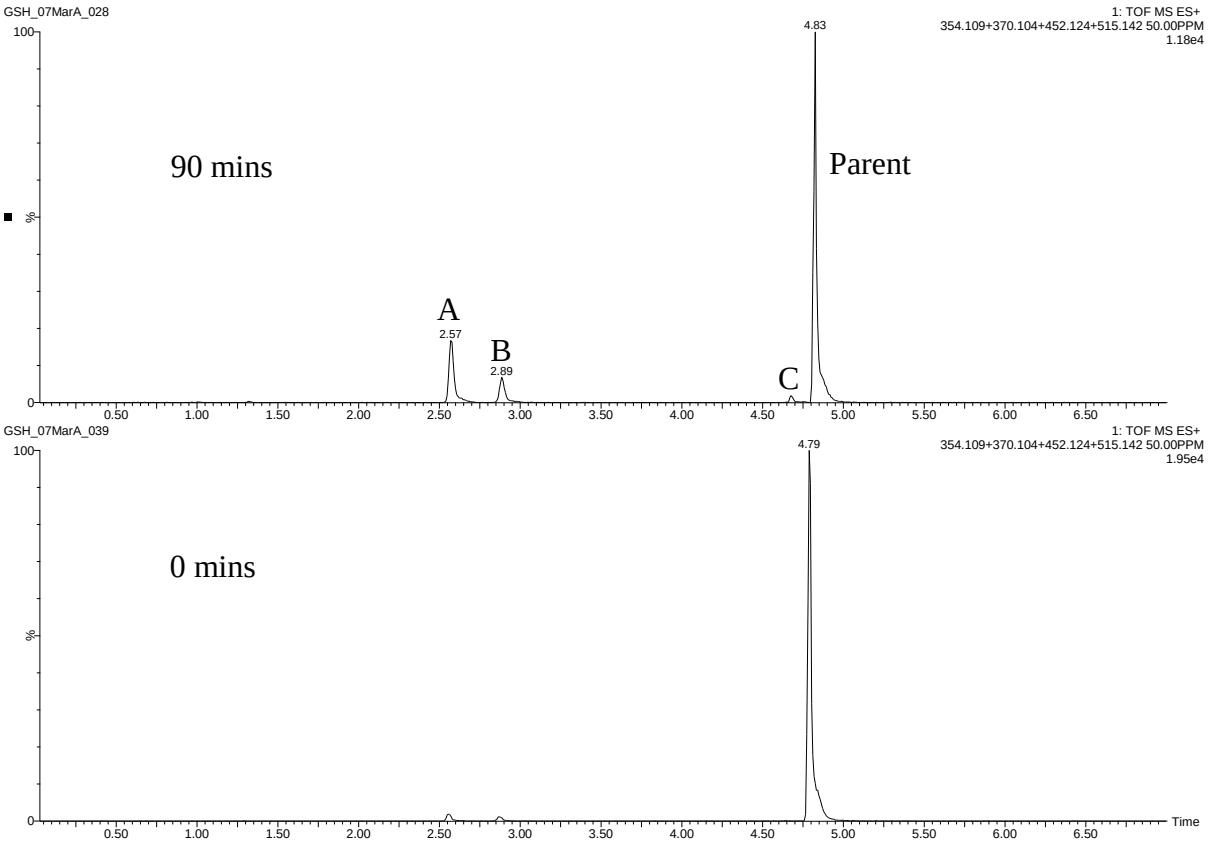


A – Cleave at amide bond

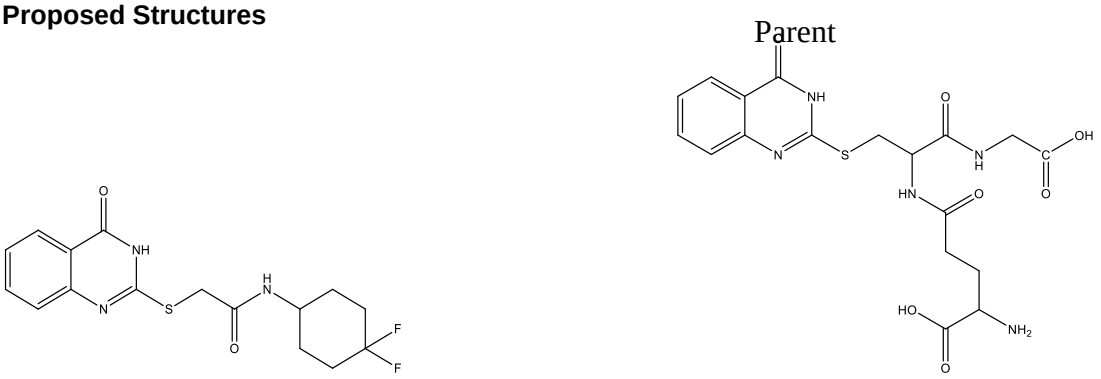


B – oxidation

***Figure S2*. Met-ID studies on compound 7 during incubation with mouse liver microsomes.**



Proposed Structures



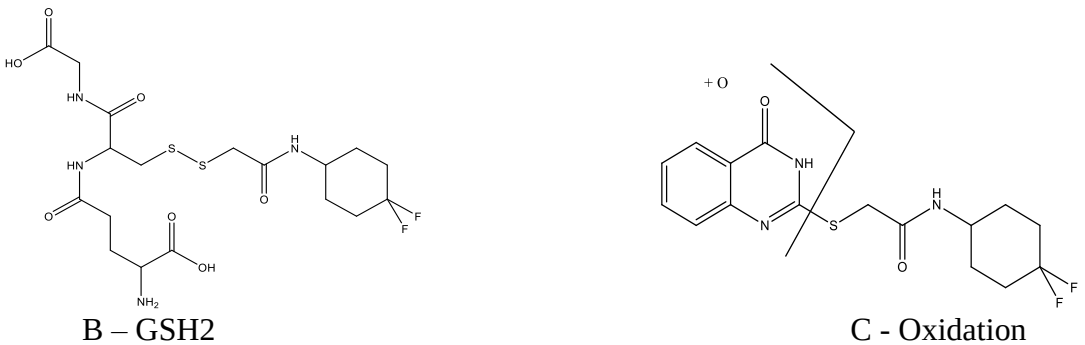


Figure S3. **Met-ID studies on compound *7* during incubation with human liver microsomes with GSH.** Metabolites A and B were detected in incubations with and without the RGS present.

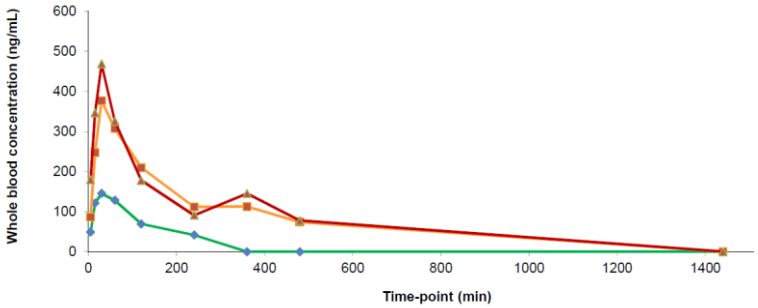


Figure S4. Once daily single rising oral dose exposure for **7** at 5, 30 and 100 mg/kg. AUC at 5, 30 and 100mg/kg are 565523, 131135 and 131878 ng.min/ml, respectively.

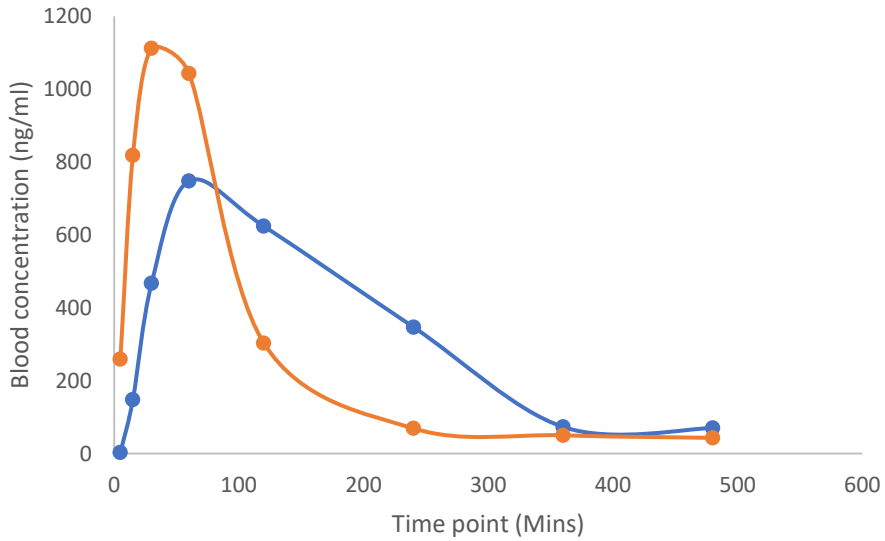


Figure S5. Comparative oral pharmacokinetics of compound **1** (blue) and compound **11** (orange) following 10mg/kg administration to female C57Bl/6 mice.

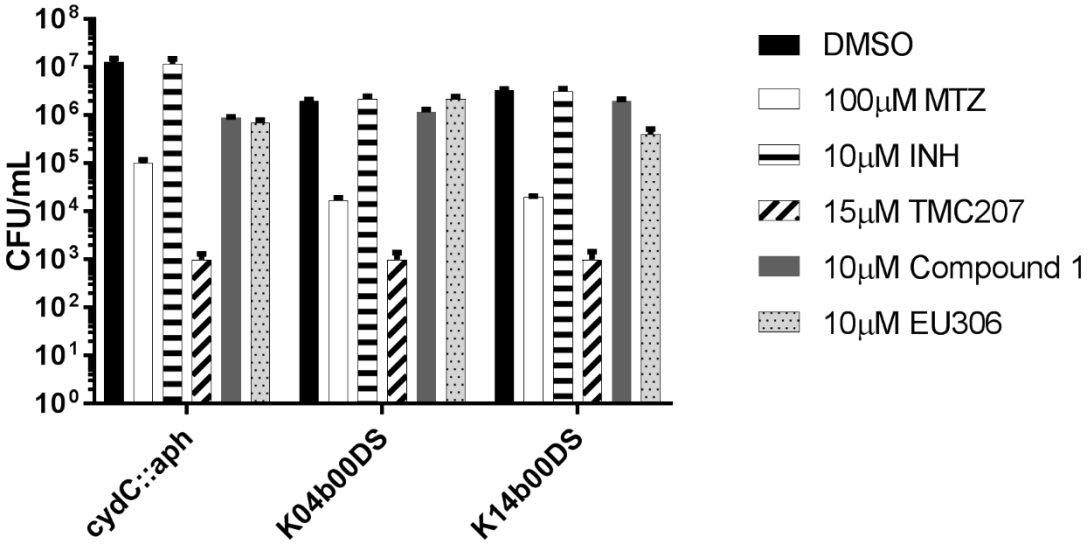


Figure S6. **Lack of anaerobic cidal activity of compound 1 against the *cydC*::*aph* deletion mutant and two drug-sensitive clinical strains of MTb (K04b00DS and K14b00DS).** Cells were anaerobically adapted to hypoxia during self-depleted oxygen consumption and at 3 weeks of adaptation, when anaerobic conditions have been achieved, cells were transferred to 24w plates containing compound or solvent (DMSO) in an anaerobic chamber maintained at 37C. After 7 days of incubation, cells were removed and aerobically plated on 7H11/OADC agar plates for CFU determination.

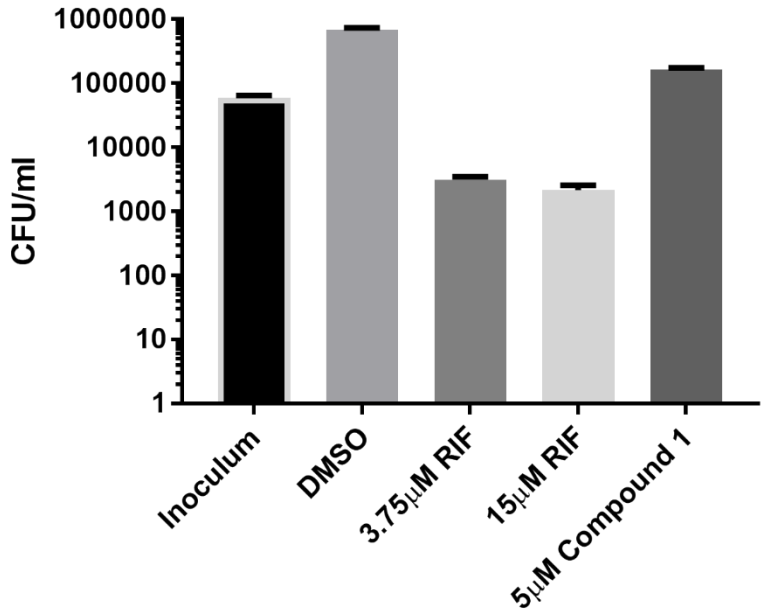


Figure S7. **Compound 1 lacks activity against MTb growing in infected macrophages.** J774 macrophages in 24w plates (500,000 cells per well) were infected with MTb H37Rv at an MOI of 1:1 for 24 hours. Infected cells were washed three times and supplemented with macrophage growth medium with compounds at the indicated concentrations or the DMSO solvent. Cell medium was replenished every 3 days and cells lyzed after 7 days of drug treatment and plated on 7H11/OADC plates for CFU enumeration.

Supplementary Tables

Table S1. Polymorphisms found among mutants selected for resistance to compound **1**.

isolate	parent strain	polymorphisms
TBK21-57-2	H37Rv	Rv3028c/*fixB*:G132D, +A in fadD26 (aa 207)
TBK21-64-2	H37Rv	A>T -44 bp upstream of *ndhA*, +A in *fadD26* (aa 207), 46kb duplication of Rv1760-Rv1798
TBK21-21-1	H37RvMA (ATCC 27294)	
TBK21-24-1	H37RvMA (ATCC 27294)	Rv0678: -T in aa 27, A>G 10 bp downstream of Rv0026
TBK21-25-2	H37RvMA (ATCC 27294)	Rv2689c:C75C
TBK21-31-2	H37RvMA (ATCC 27294)	Rv0678:Q22stop
TBK21-69-2	H37RvMA (ATCC 27294)	
TBK21-70-1	H37RvMA (ATCC 27294)	Rv0678:Q22stop
TBK21-1-2	Beijing	del of GACGGGA -47..-53 bp upstream of *ndhA*, *ppsC*: +C in aa 672, -22 bp del in *pirG*

Supplementary References

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2. Ioerger, T. R.; Feng, Y.; Ganesula, K.; Chen, X.; Dobos, K. M.; Fortune, S.; Jacobs, W. R.; Mizrahi, V.; Parish, T.; Rubin, E.; Sassetti, C.; Sacchettini, J. C., Variation among Genome Sequences of H37Rv Strains of Mycobacterium tuberculosis from Multiple Laboratories. *Journal of Bacteriology* **2010,** *192* (14), 3645-3653. DOI: 10.1128/jb.00166-10.

3. Larsen, M. H.; Biermann, K.; Tandberg, S.; Hsu, T.; Jacobs, W. R., Genetic Manipulation of Mycobacterium tuberculosis. In *Current Protocols in Microbiology*, John Wiley & Sons, Inc.: 2005. DOI: 10.1002/9780471729259.mc10a02s6.

4. Li, H.; Durbin, R., Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **2009,** *25* (14), 1754-1760. DOI: 10.1093/bioinformatics/btp324.