**Supporting Information**

# Structure-based design of MptpB inhibitors that reduce multi-drug-resistant *M. tuberculosis* survival and infection burden *in vivo*

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**Supporting Information**

**Experimental Methods.**

**Suppl. Table 1**

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## Experimental Methods (ADME determinations).

## ADME determinations for Compound 13 (data kindly supplied by LifeArc).

### LogD

LogD measurements were carried out using the shake flask method. Compound was diluted from a 10 mM DMSO stock solution into an eppendorf containing equal amounts of octanol and phosphate buffered saline (PBS) to give a final concentration of 100 µM. The tubes were shaken for 12 hours, centrifuged at 10000 rpm for 10 minutes and samples taken from the octanol and PBS layers. The samples from both layers were analysed in triplicate by LC-MS/MS (Aglient Technologies G6410 series, triple quadrupole with MM-ESI ion source) using optimised multiple reaction monitoring (MRM) scans and a standard column gradient on an Acquity UPLC BEH C8 1.7 µm column, running acetonitrile and water with 0.05% acetic acid as the mobile phase. The ratios of areas of the peaks were used to calculate the Log D in accordance with the equation:

$$\text{Log D} = \text{Log}_{10}(\text{Area-TL}/\text{Area BL})$$

### Kinetic Solubility

The kinetic solubility was measured by diluting a small amount of 10 mM DMSO stock into PBS pH7.4 in a filtration plate at a target concentration of 200 µM giving a final solution composition of 98:2 PBS:DMSO. Each compound was run in triplicate on the same plate with two standard compounds, Verapamil and Ketoconazole, included per plate. The filtration plate was shaken at 500 rpm for 90 minutes and then filtered under vacuum. The filtrate was sampled and diluted with a DMSO:PBS mixture in a flat bottomed UV plate to give a solution with a composition of PBS:DMSO 80:20. A dilution series for each compound was then created in flat bottomed UV plates in PBS:DMSO 80:20 with concentrations 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM & 6.75 µM. The UV absorbance for these solutions was read across 230-400 nM at 1 nM intervals using a TECAN Safire II plate reader and a suitable UV wavelength chosen around the UV maximum of each compound. This was used to calculate the concentration in the filtrate for each compound and hence the amount remaining in solution after 90 minutes which is reported as the kinetic solubility.

### PAMPA

PAMPA assays were carried out using a 96 well BD Biosciences pre-coated PAMPA plate with 0.4 µm polyvinylidene fluoride filter plate, precoated with structured layers of phospholipids. The compounds were run in triplicate on the same plate at a specified concentration between 200uM and 50 µM which is known to be below the previously determined kinetic solubility, the final solution having a PBS:DMSO ratio of 98:2. The compound solution was placed in the lower 'Donor' well of the plate and the upper 'Acceptor' well of the plate was filled with PBS:DMSO 98:2 buffer containing no compound following which the plate was assembled and incubated for 5 hours at room temperature. After incubation the Donor and Acceptor wells where sampled into flat bottomed plates and a six-fold 1:2 dilution series created for each compound starting at the appropriate top concentration for each compound. The UV absorbance for these solutions was read across 230-400nM at 1nM intervals using a TECAN Safire II plate reader and a suitable UV wavelength chosen around the UV maximum of each compound. This was used to calculate the concentration in the Donor and Acceptor wells for each compound. The compound permeability $P_{app}$ (nm $s^{-1}$) and %Mass retention was then calculated for each compound in accordance with the following equations:

$$C_{eq} = \frac{(C_D(t) \times V_D) + (C_A(t) \times V_A)}{V_D + V_A}$$

$$\%\ Mass\ Retention = 1 - \frac{(C_D(t) \times V_D) + (C_A(t) \times V_A)}{C_0 \times V_D}$$

A = Filter Area, $V_D$ = Donor Well Volume, $V_A$ = Acceptor Well Volume, t = incubation time (secs), $C_A(t)$ = Acceptor concentration at time t, $C_D(t)$ =Donor concentration at time t, $C_0$ = Initial Donor concentration.
No Permeability is reported for compounds where % Mass Retention exceeds 50%.

**ADME determinations for compounds in Suppl. Table 2 (Data kindly supplied by RedX Pharma Plc.).**

**logD**
The chemicals used in the assay were supplied by Sigma Aldrich. Briefly, 30 µL of a 20 mM stock solution of the compound to test was added to a vial containing 1.5 mL of pH 7.4 phosphate buffer saturated octanol and 1.5 mL of octanol saturated pH 7.4 phosphate buffer. The vial was then shaken on a reciprocating shaker at 125 rpm for 40 min. Following centrifugation to ensure phase separation, the concentration of the compound in both the octanol and buffer phases was measured by LC-UV. The Log D was then calculated as the Log of ratio of the concentration of compound in the octanol phase to that in the buffer phase.

**Solubility**
1 mL of buffer was added to 1.0 mg of compound and then incubated for 24 hr (Bioshake iQ, 650 rpm, 25 °C). Following filtration under positive pressure, the concentration of compound in solution was assessed by LC-UV in comparison to the response for a known calibration standard.

**PPB**
A known concentration of drug in plasma (20 µM) was dialysed against phosphate buffer for 18 hr at 37 °C. The concentration of compound in the protein-containing and protein-free sides of the dialysis plate were determined by LC-MS/MS. The methodology uses either 100% plasma or plasma diluted with phosphate buffer. The routine operation of the assay employed 10% plasma diluted with phosphate buffer to enable the detection of highly bound compounds. Results were corrected to reflect the result if diluted plasma was used.

**MDCK**
Briefly, monolayer integrity was measured before the assay was initiated measuring the transepithelial electrical resistance values (Ω). Compound was added to either apical (A) or basolateral (B) chamber at a final concentration of 10 µM and after 2 hr incubation at 37 °C, an aliquot from the opposite side of the monolayer was removed and quantified by LC-MS/MS. The apparent permeability ($P_{app}$) was calculated using Equation 6:

$$P_{app}\ [cm.s^{-1}] = (V/(A.C_i)) \times (C_f/T) \qquad \text{Eq.6}$$

Where V = volume of recipient chamber (mL), A = surface area of membrane insert ($cm^2$), $C_i$ = initial concentration of compound (µM), $C_f$ = final concentration of compound (µM), T = assay time (s). Efflux ratio = $P_{app\ B\ to\ A}$ / $P_{app\ A\ to\ B}$.

Data analysis was performed using Excel (Microsoft, USA).

**HLM**

Human liver microsomes were commercially obtained (Invitrogen, USA). All microsomes were stored at -80 °C. Liver microsomal stability studies were performed manually using the substrate depletion approach, drug was incubated at 37 °C with human liver microsomes (0.5 $mg.mL^{-1}$) at a final substrate concentration of 1 µM. Aliquots were removed from the incubation at 0, 5, 15, 30 and 45 min and the reaction was terminated by adding to ice-cold organic solvent. Compound concentrations were determined by LC-MS/MS analysis. The natural log of the percentage of compound remaining was plotted against each time point and the slope determined. The half-life ($t_{1/2}$) and $CL_{int}$ were calculated using Equations 1 and 2, respectively.

$t_{1/2}$ (min)=0.693/-slope Eq.1

$CL_{int}$ ($\mu L.min^{-1}.mg^{-1}$) = (LN(2)/$t_{1/2}$(min))*1000/microsomal protein ($mg.mL^{-1}$)[a] Eq. 2

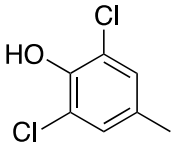
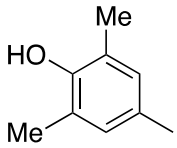
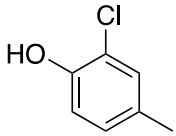
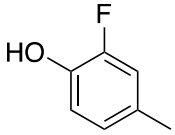
[a] or cell density ($10^6$ $cells.mL^{-1}$) or P450 concentration ($pmol.mL^{-1}$)

Percentage liver blood flow (%LBF) was determined by dividing Cl by liver blood flow (20.7 mL/min/kg). Data analysis was performed using Excel (Microsoft, USA).

**Supplementary Table 1. Molecular docking of the new series of compounds into the MptpB structure.** Free energy of binding (ΔG) values as estimated by Autodock. Experimentally determined $IC_{50}$ values (μM) towards *M. tuberculosis* phosphatases MptpB, MptpA, and the human phosphatases PTP1B and VHR are included (nd, not determined).

| | | **$IC_{50}$ (μM)** | | | |
|---|---|---|---|---|---|
| **Compound** | **ΔG (kcal/mol)** | **MptpB** | **MptpA** | **hPTP1B** | **hVHR** |
| **C1** | -9.51 | 7 | nd | 11 | nd |
| **2** | -9.43 | 17 | nd | nd | nd |
| **3** | -8.96 | 38 | nd | nd | nd |
| **4** | -10.1 | 85 | nd | nd | nd |
| **5** | -12.45 | 0.92 ± 0.03 | 33.7 ± 0.6 | 127 ± 9 | 10.5 ± 0.5 |
| **6** | -11.03 | 2.8 ± 0.3 | 74 ± 1.0 | >100 | 54.0 ± 4.6 |
| **7** | -11.33 | 2.3 ± 0.7 | 95 ± 5.0 | >100 | 59 ± 1 |
| **8** | -11.63 | 1.2 ± 0.3 | 6.5 ± 0.3 | 48 ± 3 | 12.3 ± 1.0 |
| **9** | -11.13 | 1.1 ± 0.1 | 36 ± 2 | >100 | 23.2 ± 0.3 |
| **10** | -11.72 | 0.9 ± 0.1 | 23.5 ± 0.5 | 213 ± 29 | 18.7 ± 1.5 |
| **11** | -11.36 | 1.5 ± 0.1 | 35.3 ± 3.1 | >100 | 40.2 ± 1.3 |
| **12** | -11.68 | 0.40 ± 0.05 | 30.2 ± 1.4 | 313 ± 29 | 13.0 ± 1.0 |
| **13** | -8.9 | 2.98 ± 0.27 | nd | >100 | >100 |

**Supplementary Table 2. DMPK profiles of selected MptpB inhibitors**. Data was kindly provided by Redx Pharma Plc.

| | | DMPK Profiles | | | | |
|---|---|---|---|---|---|---|
| **P2 Head** | **Cpd** | **LogD (pH 7.4)** | **Solubility (□g/ml)** | **PPB (% free)** | **MDCK $P_{app}$ (x$10^{-6}$ cm/sec) & ER** | **HLM $CL_{int}$ (% LBF)** |
|  | **5** | 0.84 | 1183 | na | 0.8, ER 1.6 | 31 |
|  | **9** | 1.37 | >1250 | 0.1 | 5.8, ER 1 | 36.4 |
|  | **10** | 1.30 | >1250 | 0.1 | 3.3, ER 0.8 | 33 |
|  | **11** | 0.67 | >1250 | 0.2 | 2.7, ER 0.7 | 47.7 |

Cpd, compound; LogD, lipophilicity; PPB, plasma protein binding; $P_{app}$, permeability in Madin-Darby Canine Kidney (MDCK) cells; ER, efflux ratio; HLM Clint, intrinsic clearance in human liver microsomes, LBF, liver blood flow; na, not available.

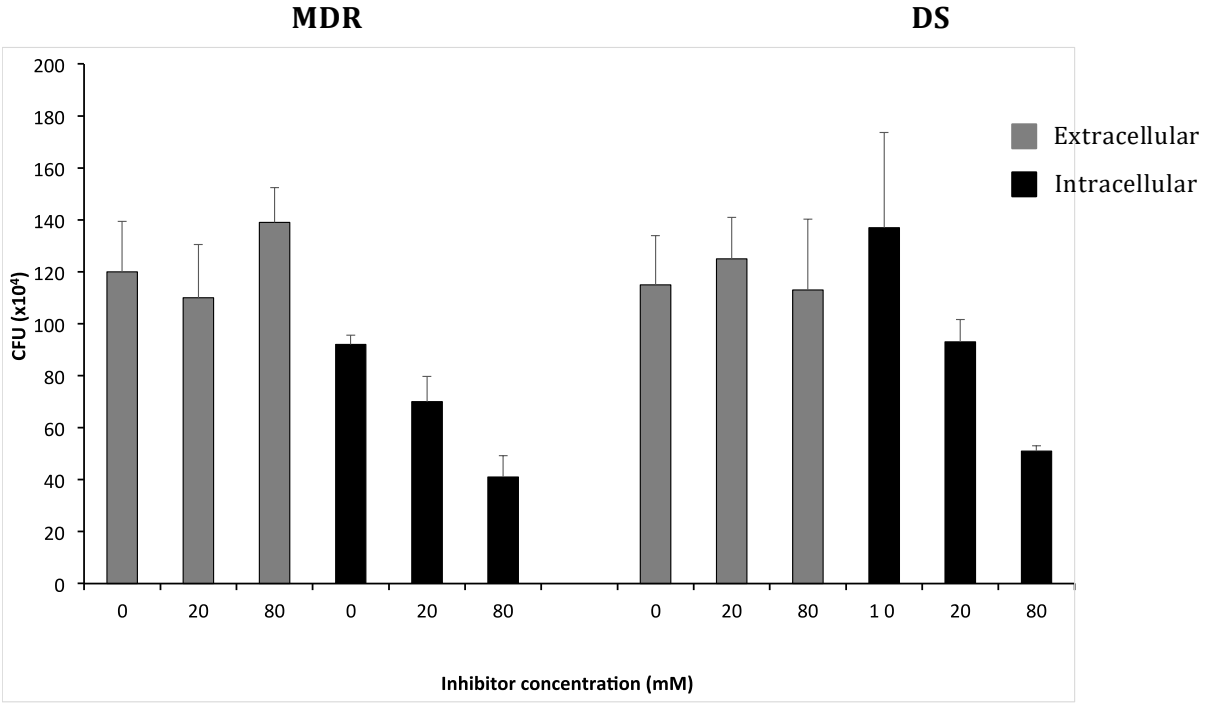
**Supplementary Table 3.** *M. tuberculosis* infection burden in the lungs and spleen for the acute and chronic guinea-pig models after 28 days of treatment with 100mg/kg of compound **13** daily or 50mg/Kg with RIF.

| Group | Lungs (log CFU) | | | Spleen (log CFU) | | |
|---|---|---|---|---|---|---|
| | **Average** | **Range** | **Δ log reduction** | **Average** | **Range** | **Δ log reduction** |
| **Acute** | | | | | | |
| Vehicle | 6.8 | 6.4 – 7.0 | **0** | 5.6 | 5.4-5.8 | **0** |
| Cpd **13** | 5.9 | 5.7 – 6.1 | **0.9** | 5.6 | 5.4-5.8 | **0** |
| RIF | BLQ | N/A | | BLQ | N/A | |
| **Chronic** | | | | | | |
| Vehicle | 5.7 | 5.6 - 6.2 | **0** | 5.2 | 4.8 - 5.3 | **0** |
| Cpd **13** | 4.7 | 4.2 - 4.9 | **1.0** | 4.2 | 4.0 - 4.6 | **1.0** |
| RIF | 3.7 | 2.8 to 4.2 | **2.0** | BLQ* | n/a | **>5.0** |

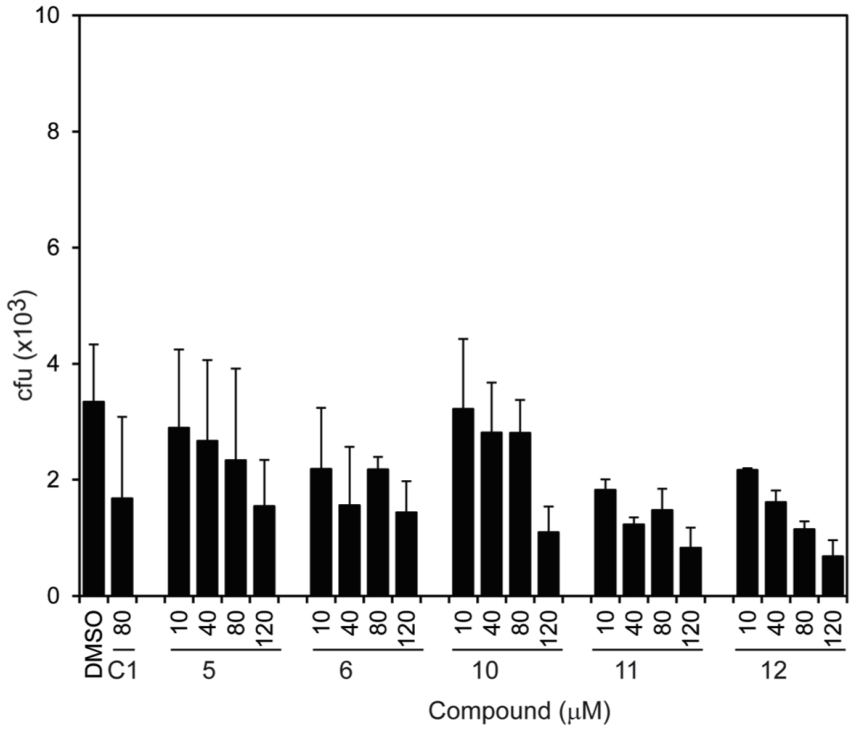
BLQ; Below limit of quantification
N/A; Not available

**Supplementary Table 4.** Guinea Pig lung and spleen weights after 28 days of treatment (Chronic infection model).

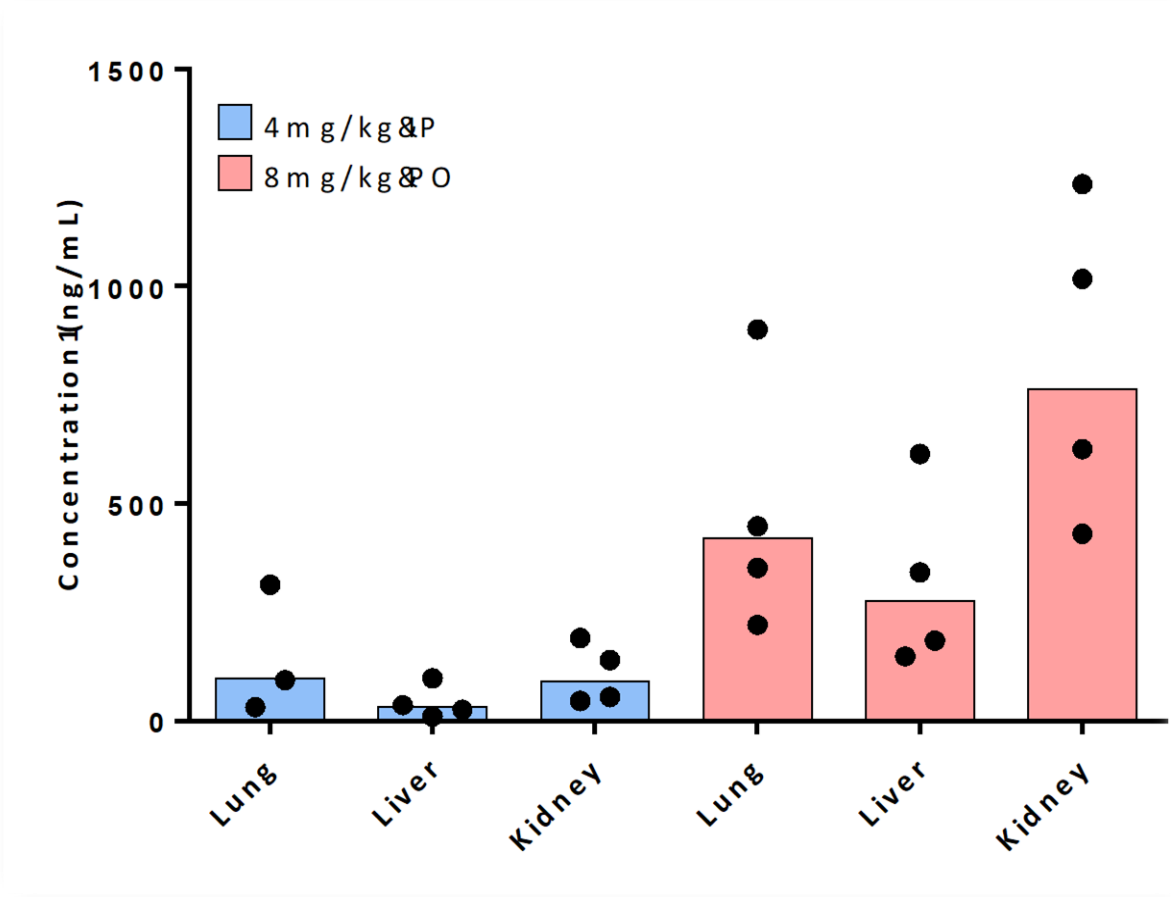
| Group | Lungs (g) | | | Spleen (g) | | |
|---|---|---|---|---|---|---|
| | **Average** | **Range** | **% change relative to Vehicle** | **Average** | **Range** | **% change relative to Vehicle** |
| Vehicle | 5.4 g | 4.4 to 6.3 | **0%** | 2.6 g | 2.1 to 3.3 | **0%** |
| Cpd **13** (100 mg/kg) | 4.4 g | 4.1 to 4.7 | **-19%** | 2.1 g | 1.2 to 3.7 | **-19%** |
| RIF (50 mg/kg) | 3.8 g | 3.4 to 4.3 | **-29%** | 0.7 g | 0.6 to 1.0 | **-73%** |




**Supplementary Figure 1. C1** compound reduces bacterial burden of a MDR strain (Beijing-w) and drug sensitive (DS) H37Rv in infected human THP1 macrophages. The effect is dose dependent with a reduction in survival up to 55% for MDR and 67% for H37Rv at 72 hours post infection (histograms in black, bars represent standard deviation of the mean). Histograms in grey show that **C1** has no effect on extracellular bacterial growth.




**Supplementary Figure 2.** Cell activity of the new series of isoxazole-based compounds results in reduction in the mycobacterial (BCG) burden of infected mouse macrophages (J774) at 24 hours post infection, compared to DMSO-treated macrophages. Plots represent the average CFUs (+SEM) per well (from a 96-well plate, see methods for details) of at least three independent experiments.




**Supplementary Figure 3.** Tissue distribution of compound **13** in guinea-pigs after administration by IP (4mg/ml) or PO (8mg/ml). Analysis was done by LC-MS on terminal 8 hour samples of lungs, liver and kidney from IP (1-4) and PO (5-8) dosed guinea-pigs.