**Supplementary Material**

**Identification and validation of the mode of action of chalcone anti-mycobacterial compounds**

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Spectral characterisation of synthesised chalcones (**1a-1o**)

3-(3,4-Dimethoxyphenyl)-1-phenylprop-2-en-1-one **1a**

IR, *ῡ* / cm-1: 3045, 2980 (C-H), 1645 (C=O).  1H NMR (600 MHz, *DMSO-d*6) ppm 3.78 (s, 3 H), 3.83 (s, 3 H), 6.98 (s, 1 H), 7.35 (d, *J*=2.02 Hz, 1 H), 7.37 (d, *J*=1.51 Hz, 1 H), 7.51 (d, *J*=2.02 Hz, 1 H), 7.54 (m, 2 H), 7.62 (m, 1 H), 7.67 (m, 1 H), 8.11 (d, *J*=1.51 Hz, 1 H), 8.12 (d, *J*=1.01 Hz, 1 H). 13C NMR (151 MHz, *DMSO-d*6)  ppm 56.14, 56.29, 111.31, 112.11, 120.15, 124.54, 128.02, 128.96, 129.26, 133.45, 138.40, 145.10, 149.56, 151.85, 189.60 (C=O). MS, m/z: 269 (M+ + H).

1-(3-Bromophenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one **1b**

IR, *ῡ* / cm-1: 3061 (C-H), 1686 (C=O), 711 (C-Br). 1H NMR (600 MHz, *DMSO-d*6)  ppm 3.79 (s, 3 H), 3.83 (s, 3 H), 7.00 (d, *J*=8.07 Hz, 1 H), 7.40 (dd, *J*=8.58, 2.02 Hz, 1 H), 7.51 (m, 2 H), 7.70 (m, 1 H), 7.78 (m, 1 H), 7.83 (m, 1 H), 8.12 (m, 1 H), 8.23 (t, *J*=1.77 Hz, 1 H). 13C NMR (151 MHz, *DMSO-d*6)  ppm 56.16, 56.34, 111.63, 112.11, 119.68, 122.81, 124.81, 127.90, 128.00, 131.36, 131.50, 136.07, 140.46, 145.98, 149.55, 152.07, 188.26 (C=O). MS, m/z: 347/349 (M+).

1-(2-Bromophenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one **1c**

IR, *ῡ* / cm-1: 2970 (C-H), 1670 (C=O). 1H NMR (600 MHz, DMSO-d6) 3.76 (s, 6 H), 6.96 (d, *J* = 8.58 Hz, 1 H), 7.12 (d, *J* = 16.15 Hz, 1 H), 7.23 - 7.24 (m, 1 H), 7.24 - 7.27 (m, 1 H), 7.35 (d, *J* = 2.02 Hz, 1 H), 7.39 - 7.44 (m, 1 H), 7.44 - 7.47 (m, 1 H), 7.48 (dd, *J* = 7.57, 1.01 Hz, 1 H), 7.68 - 7.72 (m, 1 H). 13C NMR (151 MHz, *DMSO-d*6) ppm 56.15, 56.20, 111.38, 112.12, 119.05, 124.37, 124.55, 127.38, 128.33, 129.44, 132.05, 133.59, 141.64, 147.73, 149.57, 152.14, 194.78.0 (C=O). MS, m/z: 347/349 (M+).

1-(4-Bromophenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one **1d**

IR, *ῡ* / cm-1: 3045 (C-H), 1641 (C=O). 1H NMR (600 MHz, DMSO-d6) 3.78 (s, 3 H), 3.82 (s, 3 H), 6.99 (d, *J* = 8.58 Hz, 1 H), 7.37 (dd, *J* = 8.58, 2.02 Hz, 1 H), 7.51 (d, *J* = 2.02 Hz, 1 H), 7.62 - 7.71 (m, 1 H), 7.74 (s, 1 H), 7.75 - 7.79 (m, 2 H), 8.06 (d, *J* = 8.58 Hz, 2 H). 13C NMR (151 MHz, *DMSO-d*6) ppm 56.16 (s), 56.30, 111.40, 112.11, 119.76, 124.72, 127.56, 127.92, 131.01, 132.31, 137.38, 145.66, 149.56, 152.00, 188.67 (C=O). MS, m/z: 347/349 (M+).

1-(3-Chlorophenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one **1e**

IR, *ῡ* / cm-1: 3045 (C-H), 1679 (C=O), 715 (C-Cl). 1H NMR (600 MHz, *DMSO-d*6)  ppm 3.79 (s, 3 H), 3.83 (s, 3 H), 7.00 (d, *J*=8 Hz, 1 H), 7.39 (dd, *J*=8, 2 Hz, 1 H), 7.52 (d, *J*=2 Hz, 1 H), 7.58 (t, *J*=8 Hz, 1 H), 7.70 (m, 2 H), 7.79 (m, 1 H), 8.08 (m, 1 H), 8.12 (t, *J*=2 Hz, 1 H). 13C NMR (151 MHz, *DMSO-d*6) ppm 56.16, 56.34, 111.57, 112.11, 119.69, 124.83, 127.63, 127.90, 128.52, 131.26, 133.17, 134.31, 140.27, 145.98, 149.56, 152.07, 188.31 (C=O). MS, m/z: 303/305 (M+).

1-(2-Chlorophenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one **1f**

IR, *ῡ* / cm-1: 2983 (C-H), 1661 (C=O), 710 (C-Cl). 1H NMR (600 MHz, *DMSO-d*6) ppm 3.76 (s, 3 H), 3.77 (s, 3 H), 6.96 (d, *J*=9 Hz, 1 H), 7.14 (m, 1 H), 7.25 (dd, *J*=9, 2 Hz, 1 H), 7.28 (m, 1 H), 7.36 (d, *J*=2 Hz, 1 H), 7.45 (m, 1 H), 7.50 (m, 2 H), 7.55 (m, 1 H). 13C NMR (151 MHz, *DMSO-d*6) ppm 56.15, 56.20, 111.39, 112.12, 124.37, 124.74, 127.37, 127.88, 129.56, 130.30, 130.53, 132.03, 139.60, 147.53, 149.57, 152.14, 193.87 (C=O). MS, m/z: 303/305 (M+).

1-(4-Chlorophenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one **1g**

IR, *ῡ* / cm-1: 2980 (C-H)**,** 1675 (C=O). 1H NMR (600 MHz, *DMSO-d*6)  ppm 3.79 (s, 3 H), 3.82 (s, 3 H), 7.00 (d, *J*=9 Hz, 1 H), 7.37 (dd, *J*=9, 2 Hz, 1 H), 7.51 (d, *J*=2 Hz, 1 H), 7.68 (m, 1 H), 7.78 (m, 1 H), 8.14 (m, 2 H). 13C NMR (151 MHz, *DMSO-d*6)  ppm 56.16, 56.30, 111.39, 112.11, 119.79, 124.72, 127.93, 129.36, 130.89, 137.05, 138.39, 145.62, 149.56, 151.99, 188.47 (C=O). MS, m/z: 303/305 (M+).

3-(3,4-Dimethoxyphenyl)-1-(4-fluorophenyl)prop-2-en-1-one **1h**

IR, *ῡ* / cm-1: 2937 (C-H), 1653 (C=O). 1H NMR (600 MHz, *DMSO-d*6) ppm 3.78 (s, 3 H), 3.83 (s, 3 H), 6.99 (d, *J*=9 Hz, 1 H), 7.36 (m, 3 H), 7.51 (d, *J*=2 Hz, 1 H), 7.67 (m, 1 H), 7.80 (m, 1 H), 8.21 (m, 2 H). 13C NMR (151 MHz, *DMSO-d*6)ppm 56.15, 56.30, 111.37, 112.11, 116.19, 116.33, 119.87, 124.61, 127.97, 131.91, 131.97, 145.27, 149.56, 151.91, 188.09 (C=O). MS, m/z: 287 (M+ + H).

3-(3,4-Dimethoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one **1i**

IR, *ῡ* / cm-1: 2895 (C-H), 1688 (C=O). 1H NMR (600 MHz, *DMSO-d*6) ppm 3.78 (s, 3 H), 3.83 (s, 3 H), 3.83 (s, 3 H), 6.98 (d, *J*=9 Hz, 1 H), 7.05 (m, 2 H), 7.34 (dd, *J*=8, 2 Hz, 1 H), 7.49 (d, *J*=2 Hz, 1 H), 7.63 (d, *J*=16 Hz, 1 H), 7.79 (d, *J*=15 Hz, 1 H), 8.13 (m, 2 H). 13C NMR (151 MHz, *DMSO-d*6)  ppm 56.09, 56.13, 56.28, 111.30, 112.11, 114.47, 120.13, 124.26, 128.18, 131.24, 131.33, 144.17, 149.54, 151.66, 163.60, 187.81 (C=O). MS, m/z: 299 (M+ + H).

3-(3,4-Dimethoxyphenyl)-1-(p-tolyl)prop-2-en-1-one **1j**

IR, *ῡ* / cm-1: 3062 (C-H), 1699 (C=O). 1H NMR (600 MHz, *DMSO-d*6) ppm 2.37 (s, 3 H), 3.78 (s, 3 H), 3.83 (s, 3 H), 6.99 (d, *J*=8 Hz, 1 H), 7.35 (m, 3 H), 7.50 (d, *J*=2 Hz, 1 H), 7.65 (d, *J*=15 Hz, 1 H), 7.78 (d, *J*=15 Hz, 1 H), 8.03 (m, 2 H). 13C NMR (151 MHz, *DMSO-d*6)  ppm 21.72, 56.13, 56.30, 111.30, 112.10, 120.15, 124.42, 128.09, 129.12, 129.81, 135.86, 143.83, 144.71, 149.56, 151.77, 189.00 (C=O). MS, m/z: 283 (M+ + H).

1-([1,1'-Biphenyl]-4-yl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one **1k**

IR, *ῡ* / cm-1: 2939 (C-H), 1678 (C=O). 1H NMR (600 MHz, *DMSO-d*6)  ppm 3.79 (s, 3 H), 3.84 (s, 3 H), 7.00 (d, *J*=8 Hz, 1 H), 7.40 (m, 2 H), 7.51 (m, 3 H), 7.74 (m, 3 H), 7.84 (m, 3 H), 8.22 (m, 2 H). 13C NMR (151 MHz, *DMSO-d*6)  ppm 56.16, 56.31, 111.36, 112.13, 120.17, 124.57, 127.46, 127.56, 128.07, 128.90, 129.64, 129.74, 137.23, 139.53, 144.86, 145.04, 149.58, 151.87, 189.00 (C=O). MS, m/z: 345 (M+ + H).

3-(3,4-Dimethoxyphenyl)-1-(3-nitrophenyl)prop-2-en-1-one **1l**

IR, *ῡ* / cm-1: 2945 (C-H), 1676 (C=O) 1535, 1355 (NO2). 1H NMR (600 MHz, *DMSO-d*6) ppm 3.80 (s, 3 H), 3.83 (s, 3 H), 7.02 (d, *J*=9 Hz, 1 H), 7.43 (dd, *J*=8, 2 Hz, 1 H), 7.54 (d, *J*=2 Hz, 1 H), 7.77 (m, 1 H), 7.85 (m, 2 H), 8.46 (m, 1 H), 8.58 (dt, *J*=8, 1 Hz, 1 H), 8.75 (m, 1 H). 13C NMR (151 MHz, *DMSO-d*6) ppm 56.19, 56.35, 111.72, 112.15, 119.53, 123.22, 124.99, 127.65, 127.80, 131.08, 135.18, 139.65, 146.72, 148.76, 149.57, 152.24, 187.94 (C=O). MS, m/z: 314 (M+ + H).

3-(3,4-Dimethoxyphenyl)-1-(o-tolyl)prop-2-en-1-one **1m**

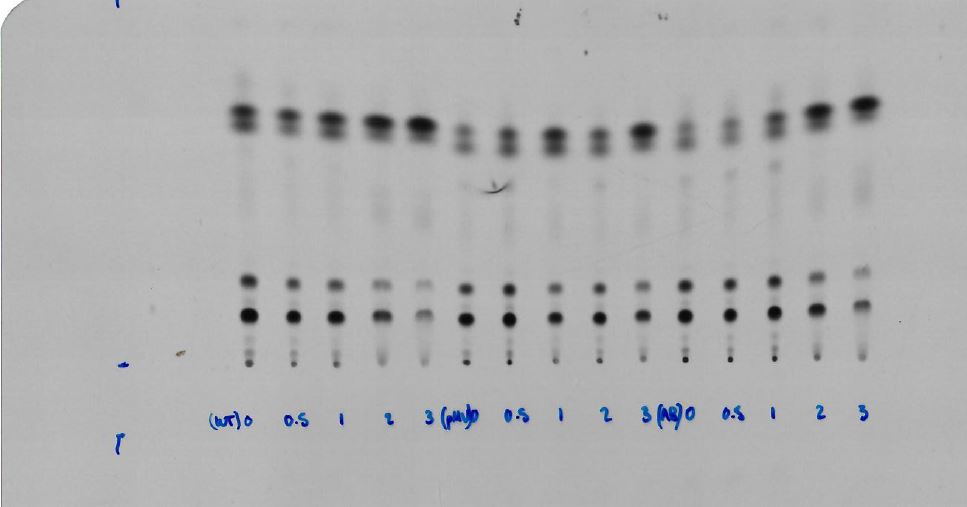
IR, *ῡ* / cm-1: 2980 (C-H)**,** 1675 (C=O). 1H NMR (600 MHz, *DMSO-d*6)  ppm 2.32 (s, 3 H), 3.77 (s, 3 H), 3.77 (s, 3 H), 6.96 (d, *J*=8 Hz, 1 H), 7.24 (m, 2 H), 7.30 (m, 2 H), 7.35 (m, 1 H), 7.39 (m, 2 H), 7.53 (m, 1 H). 13C NMR (151 MHz, *DMSO-d*6) ppm 20.34, 56.13, 56.20, 111.25, 112.11, 124.16, 124.77, 126.21, 127.67, 128.50, 130.85, 131.59, 136.53, 139.81, 146.01, 149.55, 151.85, 195.85 (C=O). MS, m/z: 283 (M+ + H).

3-(3,4-Dimethoxyphenyl)-1-(4-nitrophenyl)prop-2-en-1-one **1n**

IR, *ῡ* / cm-1: 2940 (C-H), 1666 (C=O) 1545, 1345 (NO2). 1H NMR (600 MHz, *DMSO-d*6)  ppm 3.79 (s, 3 H), 3.83 (s, 3 H), 7.01 (d, *J*=9 Hz, 1 H), 7.40 (dd, *J*=8, 2 Hz, 1 H), 7.53 (d, *J*=2 Hz, 1 H), 7.73 (m, 1 H), 7.80 (m, 1 H), 8.31 (m, 2 H), 8.34 (m, 2 H). 13C NMR (151 MHz, *DMSO-d*6) ppm 56.16, 56.28, 111.41, 112.08, 119.87, 124.36, 125.07, 127.74, 130.30, 143.27, 146.77, 149.54, 150.23, 152.24, 188.72 (C=O). MS, m/z: 314 (M+ + H).

1-(2,4-Dichlorophenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one **1o**

IR, *ῡ* / cm-1: 2850 (C-H), 1679 (C=O). 1H NMR (600 MHz, *DMSO-d*6)  ppm 3.76 (s, 3 H), 3.77 (s, 3 H), 6.97 (d, *J*=9 Hz, 1 H), 7.13 (d, *J*=16 Hz, 1 H), 7.29 (m, 2 H), 7.36 (d, *J*=2 Hz, 1 H), 7.54 (m, 2 H), 7.74 (m, 1 H). 13C NMR (151 MHz, *DMSO-d*6)  ppm 56.16, 56.20, 111.53, 112.13, 124.45, 124.53, 127.34, 128.14, 130.11, 130.98, 131.58, 135.75, 138.35, 148.21, 149.55, 152.23, 193.07 (C=O). MS, m/z: 337 (M+).



**C**

**A**

**B**

**Figure S-1:** Inhibition of mycolic acid biosynthesis by Chalcone **1a**. **A)** *Mycobacterium bovis* BCG(wild-type) cultures, B) *Mycobacterium bovis* BCG(with empty vector PMv261) cultures, C) *Mycobacterium bovis* BCG(HadAB over expressor) cultures, labelled with [14C]-acetate were exposed to increasing concentrations of **1a**. The α- and k-MAMEs and FAMEs were extracted and analysed by autoradiography-TLC. From left to right: Untreated, treated with 0.5x MIC, 1x MIC, 2x MIC and 3x MIC of **1a**. The data for B and C given in supplementary data are not included in the manuscript and will be discussed in future communications.

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**Figure S-2:** Investigation of the impact of HadABC, KasA, KasB, FabA and MabA overexpression on sensitivity to compound **1a**. Using *M. bovis* BCG containing a constitutive plasmid (pMV261) expressing HadABC, KasA, KasB, FabA and MabA, IC50 was compared to an empty vector (EV) control. The 0 and 7days means the absorbance taken at the start of the experiment and after 7 days of incubation. None of the overexpressors showed increased resistance as compared to the control vector.



**Figure S-3:** Dose dependent (0-100 µg/ml) reduction in optical density by chalcone inhibitor **1a,** INH and METagainst non-replicating persistent (NRP) *Mycobacterium bovis* BCG using the Wayne model. An endpoint ANOVA was performed, and *p* values determined as 0.0153 and 0.0311 for INH and MET respectively, demonstrating a significance in dose-dependent inhibition. INH was not found to be significant (*p* = 0.7201). The activity of compound **1a** against NRP *M. bovis* BCG suggests the direct inhibition of InhA, as INH requires activation by KatG and therefore lacks activity under NRP conditions, however this requires further validation by assessing reduction in colony forming units.

**Intrinsic tryptophan fluorescence (ITF) assay:**

In this assay, the ligand concentration (**1a** and Triclosan) was increased and the binding affinity to the InhA was measured in fluorescence. From the experiment, Kd (ligand concentration that binds to half the receptor sites at equilibrium) and Bmax (maximum number of binding sites) were determined as shown in the Table 2. The value of R2 quantifies the goodness of fit and is a fraction between 0.0 to 1.0. The higher value indicates that the model fits the data better.

**Table S-1: Analysis of Non-linear regression (curve fit) of ITF**

|  |  |  |
| --- | --- | --- |
| **One site -- Specific binding** | **1a** | **Triclosan** |
| **Best-fit values** | | |
| **Bmax** | 3454 | 2046 |
| **Kd** | 10.29 | 11.65 |
| **Std. Error** | | |
| **Bmax** | 263.3 | 507.6 |
| **Kd** | 2.515 | 8.759 |
| **95% Confidence Intervals** |  |  |
| **Bmax** | 2921 to 3986 | 1020 to 3072 |
| **Kd** | 5.209 to 15.37 | -6.051 to 29.35 |
| **Goodness of Fit** | | |
| **Degrees of Freedom** | 40 | 40 |
| **R²** | 0.8749 | 0.4781 |
| **Absolute Sum of Squares** | 6.683e+006 | 2.096e+007 |
| **Sy.x** | 408.7 | 723.8 |
| **Number of points** | | |
| **Analyzed** | 42 | 42 |