1 **Supplemental Material**

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3 ***Mycobacterium tuberculosis* Gyrase Inhibitors (MGI) as a New Class of**

4 **Antitubercular Drugs**

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15

16 ***Compound synthesis***

17 **General information**

18 All commercially available reagents and solvents were used without further purification.

19 When reactions were performed under microwave conditions, a Biotage Initiator was used.

20 Automated flash chromatography was performed on a Biotage FlashMaster II system with

21 peak detection at 254 nm. All products were obtained as amorphous solids and melting

22 points were not measured. 1H NMR spectra were recorded at 400 MHz on a Bruker

23 Ultrashield DPX 400 spectrometer. Chemical shifts (δ) are given in ppm relative to the

24 solvent reference as an internal standard (d6-DMSO, δ = 2.50 ppm; CDCl3, δ = 7.27 ppm;

25 CD3OD, δ = 3.31 ppm). Data are reported as follows: chemical shift (multiplicity (s for singlet, d for doublet, t for triplet, m for multiplet, br for broad), integration, coupling constant(s) in Hz). HPLC–MS analyses were conducted on an Agilent 1100 instrument equipped with a Sunfire C18 column (30 mm x 2.1 mm i.d., 3.5 mm packing diameter) at 40 ºC coupled with a Waters ZMD2000 mass spectrometer; the method of ionization was alternate-scan positive and negative electrospray. Semi-preparative chiral HPLC were conducted on an Agilent 1100 instrument equipped with a Chiralpack IC column (250 x 20 mm). Preparative chiral HPLC were conducted on a Varian SD-2 prep HPLC instrument equipped with a Chiralpak IC column (250 x 50mm i.d, 20 um). Compounds had purity of >95%, as determined by HPLC and 1H NMR analysis.

35 **8-Bromo-2,7-dimethoxy-1,5-naphthyridine (1).** 8-Bromo-7-fluoro-2-methoxy-1,5-naphthyridine (commercial source) (5.14g, 20 mmol) was stirred in methanol (60 mL) at rt and a solution of sodium methoxide in methanol (10.8g in 44 mL of methanol) was added. The mixture was heated at 55ºC for 4h. The mixture was allowed to cool to rt, then was diluted with water and brine, and extracted with DCM. The DCM extracts were combined, dried over anhydrous magnesium sulphate, filtered and evaporated under reduced pressure to give 8-bromo-2,7-dimethoxy-1,5-naphthyridine as a cream solid (5.05g, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.54 (s, 1H), 8.14 (d, 1H, J=0.03), 7.02 (d, 1H, J=0.03), 4.16 (s, 6H). [ES+ MS] m/z 269/271 (M+H)+.

44 **2,7-Dimethoxy-1,5-naphthyridine (2).** 8-Bromo-2,7-dimethoxy-1,5-naphthyridine (1.7g, 6.3mmol) was stirred in methanol (68 mL) and DCM (10 mL) with sodium hydrogen carbonate (1.16g, 13.8mmol) and 5% palladium on activated carbon, wet (408 mg), and the resulting suspension was hydrogenated at 30 psi of hydrogen pressure overnight. The mixture was filtered. The filtrate was concentrated under reduced pressure and the residue

49 treated with DCM and water. The organic phase was separated, dried over anhydrous
50 sodium sulphate, filtered and evaporated under reduced pressure to give 2,7-dimethoxy-1,5-
51 naphthyridine as a white solid (990mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.53 (s,
52 1H), 8.14 (d, 1H, J=0.03), 7.46 (s, 1H), 6.97 (d, 1H, J=0.03), 4.07 (s, 3H), 3.96 (s, 3H).
53 [ES+ MS] m/z 191 (M+H)+.

54 **7-Methoxy-1,5-naphthyridin-2(1H)-one (3).** A solution of 2,7-dimethoxy-1,5-
55 naphthyridine (2.05g, 10.8mmol) in 6N HCl (17 mL) was refluxed for 6h. Mixture was
56 cooled down and water (17 mL) was added and the pH of the suspension was adjusted to
57 *ca.* pH 6 by addition of solid sodium carbonate and finally 10% sodium hydrogen carbonate
58 solution. The mixture was then filtered and rinsed with water. Finally, it was dried into the
59 oven at 40ºC under vacuum to give 7-methoxy-1,5-naphthyridin-2(1H)-one (1.85g, 97%).
60 %). ^{1}H NMR (400 MHz, d_6-DMSO) δ ppm: 11.77 (s, 1H), 8.20 (s, 1H), 7.85 (d, 1H,
61 J=0.03), 7.13 (s, 1H), 6.53 (d, 1H, J=0.03), 3.86 (s, 3H). [ES+ MS] m/z 177 (M+H)+.

62 **1-Allyl-7-methoxy-1,5-naphthyridin-2(1H)-one (4).** 7-Methoxy-1,5-naphthyridin-2(1H)-
63 one (1.83g, 10.4mmol) was suspended in a mixture of DME (64 mL) and DMF (16 mL)
64 under argon at 0 ºC, and the stirred suspension was treated with sodium hydride (832mg,
65 60% suspension in oil, 20.8mmol) and stirred at 0 ºC for 45min. Lithium bromide (2.25g,
66 26mmol) was then added. The reaction was stirred at rt for 30min. Allyl iodide (3.47g,
67 20.8mmol) was added and the mixture was heated at 75 ºC overnight. The mixture was
68 concentrated under vacuum and the residue was diluted with water and ethyl acetate. The
69 organic extract was dried over sodium sulphate, filtered and evaporated under reduced
70 pressure. The residue was purified by chromatography on silica gel using a 0-100%
71 hexane/ethyl acetate gradient to provide the desired compound as a yellow solid (1.3g,

72 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.28 (s, 1H), 7.88 (d, 1H, J=0.03), 7.03 (s, 1H),
73 6.79 (d, 1H, J=0.03), 6.00-5.88 (m, 1H), 5.26 (d, 1H, J=0.04), 5.11 (d, 1H, J=0.05), 4.91 (d,
74 2H, J=0.02), 3.94 (s, 3H). [ES+ MS] m/z 217 (M+H)+.

75 **2-(7-Methoxy-2-oxo-1,5-naphthyridin-1(2H)-yl)acetaldehyde (5).** 1-Allyl-7-methoxy-
76 1,5-naphthyridin-2(1H)-one (1.1g, 5.1mmol) was dissolved in 1,4-dioxane (24 mL) and
77 water (12 mL). Sodium periodate (2.5g, 11.7mmol) and supported OsO4 (0.8g, 5% w). The
78 mixture was stirred at rt for 6h. Mixture was filtered and the polymer washed with water
79 and THF. Aqueous mixture was extracted with 25% DCM/MeOH. The combined organic
80 extracts were combined, dried, filtered and evaporated under reduced pressure to give 2-(7-
81 methoxy-2-oxo-1,5-naphthyridin-1(2H)-yl)acetaldehyde as a pale yellow (1.05g, 95%). %).
82 ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 9.72 (s, 1H), 8.30 (d, 1H), 7.94 (d, 1H), 6.81 (d, 1H),
83 6.72 (bd, 1H), 5.11 (s, 2H), 3.93 (s, 3H). [ES+ MS] m/z 219, 251 (M+H)+ (consistent with
84 the hemiacetal structure).

85 **tert-Butyl (1-(2-(7-methoxy-2-oxo-1,5-naphthyridin-1(2H)-yl)ethyl)piperidin-4-
86 yl)carbamate (6).** To a mixture of 2-(7-methoxy-2-oxo-1,5-naphthyridin-1(2H)-
87 yl)acetaldehyde (1.4g, 6.4mmol) in DCM/MeOH (50/2.5 mL) was added tert-butyl
88 piperidin-4-ylcarbamate (1.3g, 6.4mmol). After stirring for 1h, sodium
89 triacetoxyborohydride (4.1g, 19.2 mmol) was added. The reaction was stirred for 1h before
90 addition of water and saturated sodium hydrogen carbonate solution (50 mL). The reaction
91 was extracted with 20% DCM/MeOH. The combined organic phases were dried, filtered
92 and concentrated under vacuum. The residue was purified by chromatography on silica gel
93 using a 0-20% DCM/MeOH to provide the desired compound as a yellow solid (1.56g,
94 61%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.27 (d, 1H), 7.83 (d, 1H), 7.17 (bs, 1H), 6.73

95 (d, 1H), 4.44 (bs, 1H), 4.35 (t, 2H), 3.97 (s, 3H), 3.47 (bs, 1H), 2.94 (bd, 2H), 2.64 (t, 2H),
96 2.26 (t, 2H), 1.95 (bd, 2H), 1.70 (bs, 2H), 1.44 (s, 9H).
97 **1-(2-(4-Aminopiperidin-1-yl)ethyl)-7-methoxy-1,5-naphthyridin-2(1H)-one**
98 **dihydrochloride (7).** To a solution of tert-butyl (1-(2-(7-methoxy-2-oxo-1,5-naphthyridin-
99 1(2H)-yl)ethyl)piperidin-4-yl)carbamate (1.55g, 3.86mmol) in DCM (25 mL) was added
100 HCl (4M solution in 1,4-dioxan, 8 mL). After stirring overnight, the obtained solid was
101 filtered and dried to give 1.5g (95%) of the title compound.
102 **1-(2-(4-(((2,3-Dihydro-[1,4]dioxino[2,3-c]pyridin-7-yl)methyl)amino)piperidin-1-**
103 **yl)ethyl)-7-methoxy-1,5-naphthyridin-2(1H)-one (8).** A suspension of 1-(2-(4-
104 aminopiperidin-1-yl)ethyl)-7-methoxy-1,5-naphthyridin-2(1H)-one dihydrochloride (1.09g,
105 2.65mmol) in DCM (40 mL) and MeOH (3.5 mL) was treated with triethylamine (1.22 mL,
106 8.73mmol) and stirred for 15min at rt before the addition of 2,3-dihydro-[1,4]dioxino[2,3-
107 c]pyridine-7-carbaldehyde (commercial source) (0.42g, 2.51mmol) in one portion. The
108 mixture was stirred for 30min at rt and sodium triacetoxyborohydride (1.68g, 7.94mmol)
109 was added in one portion. The reaction was stirred for 2.5h before addition of water and
110 saturated sodium hydrogen carbonate solution (150 mL). The reaction was extracted with 2-
111 20% DCM/MeOH. The combined organic phases were dried, filtered and concentrated
112 under vacuum. The residue was purified by chromatography on silica gel using a 0-20%
113 DCM/MeOH to provide the desired compound as a yellow solid (0.79g, 66%).
114 **1-(2-(4-(((2,3-Dihydro-[1,4]dioxino[2,3-c]pyridin-7-yl)methyl)amino)piperidin-1-**
115 **yl)ethyl)-7-methoxy-1,5-naphthyridin-2(1H)-one hydrochloride (9).** 1-(2-(4-(((2,3-
116 Dihydro-[1,4]dioxino[2,3-c]pyridin-7-yl)methyl)amino)piperidin-1-yl)ethyl)-7-methoxy-
117 1,5-naphthyridin-2(1H)-one (0.79g, 2.21mmol) was converted to the HCl salt by dissolving
118 the obtained free base in DCM (10 mL) and MeOH (5 mL) and adding HCl (0.6 mL, 4M in

119 1,4-dioxane). The white suspension was filtered at rt for 15min before evaporating in
120 vacuum. The white solid was suspended in ^{t}BuOMe (20 mL) and filtered to yield the title
121 compound as a white solid (0.775g). ^{1}H NMR (400 MHz, d_6-DMSO) δ ppm: 11.38 (bs,
122 1H), 9.59 (bs, 2H), 8.31 (s, 1H), 8.16 (s, 1H), 7.91 (d, 1H, J = 0.04), 7.69 (bs, 1H), 7.19 (s,
123 1H), 6.69 (d, 1H, J = 0.04), 4.68 (bs, 2H), 4.38-4.32 (m, 4H), 4.18 (s, 2H), 4.04 (s, 3H),
124 3.75 (bs, 2H), 3.28 (bs, 3H), 3.05 (bs, 2H), 2.32 (bs, 2H), 2.06 (bs, 2H).

125 **1-Allyl-1,5-naphthyridin-2(1H)-one (10).** 1,5-naphthyridin-2(1H)-one (commercial
126 available) (500mg, 3.42mmol) and potassium carbonate (1.5g, 10.8mmol) were suspended
127 in DMF (10 mL). Allyl bromide (1.24g, 10.2mmol) was added in one portion and the
128 resulting mixture was stirred overnight. The mixture was diluted with water and DCM. The
129 organic extract was dried over sodium sulphate, filtered and evaporated under reduced
130 pressure. The residue was purified by chromatography on silica gel using a 0-10%
131 DCM/MeOH gradient to provide the desired compound (425mg, 67%). [ES+ MS] m/z 187
132 (M+H)+.

133 **2-(2-Oxo-1,5-naphthyridin-1(2H)-yl)acetaldehyde (11).** 1-Allyl-1,5-naphthyridin-2(1H)-
134 one (400mg, 2.15mmol) was dissolved in 1,4-dioxane (10 mL) and water (5 mL) and the
135 solution was cooled to 0ºC. Sodium periodate (1.06g, 4.99mmol) was added followed by
136 OsO_4 (1.60 ml, 4% aqueous solution). The mixture was allowed to warm to room
137 temperature and stirred overnight. Mixture was concentrated to dryness and dissolved in
138 water. The aqueous layer was extracted with 10% DCM/MeOH. The combined organic
139 extracts were combined, dried, filtered and evaporated under reduced pressure to give the
140 title compound as a mixture with methyl hemiacetal (310mg, 77%).

141 **tert-Butyl (1-(2-(2-oxo-1,5-naphthyridin-1(2H)-yl)ethyl)piperidin-4-yl)carbamate (12).**
142 To a mixture of 2-(2-oxo-1,5-naphthyridin-1(2H)-yl)acetaldehyde (4.5g, 23.9mmol) and

143 tert-butyl piperidin-4-ylcarbamate (4.785g, 23.9mmol) in DCE (150 mL) was added sodium
144 triacetoxyborohydride (7.08g, 33.4mmol). The reaction was stirred overnight before
145 addition of saturated sodium hydrogen carbonate solution (70 mL). The reaction was
146 extracted with ethyl acetate. The combined organic phases were dried, filtered and
147 concentrated under vacuum. The residue was purified by chromatography on silica gel
148 using a 0-20% DCM/MeOH to provide the desired compound as a yellow foam (5.94g,
149 67%). [ES+ MS] m/z 373 (M+H)+.

150 **1-(2-(4-Aminopiperidin-1-yl)ethyl)-1,5-naphthyridin-2(1H)-one (13).** To a solution of
151 tert-Butyl (1-(2-(2-oxo-1,5-naphthyridin-1(2H)-yl)ethyl)piperidin-4-yl)carbamate (5.7g,
152 15.3mmol) in DCM (20 mL) was added trifluoroacetic acid (20 mL). After stirring 2 h, the
153 mixture was concentrated to dryness and basified until pH 12 with aqueous solution of
154 NaOH. The organic layer was extracted with DCM to give the title compound (191mg,
155 88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.55 (d, 1H), 7.90 (d, 1H), 7.77 (d, 1H), 7.48-
156 7.44 (m, 1H), 6.91 (d, 1H), 4.38 (t, 2H), 2.99-2.94 (m, 2H), 2.73-2.62 (m, 3H), 2.18 (dt,
157 2H), 1.87-1.76 (m, 2H), 1.43-1.30 (m, 2H), 1.25-1.13 (m, 2H).

158 **1-(2-(4-((3,4-Dichlorobenzyl)amino)piperidin-1-yl)ethyl)-1,5-naphthyridin-2(1H)-one**
159 **(14).** A mixture of 1-(2-(4-aminopiperidin-1-yl)ethyl)-1,5-naphthyridin-2(1H)-one (60mg,
160 0.22mmol) and 3,4-dichlorobenzaldehyde (commercial source) (38.28mg, 0.22mmol) in
161 DCE (5 mL) was stirred at rt for 1h, and sodium triacetoxyborohydride (65mg, 0.31mmol)
162 was added. The reaction was stirred overnight before addition of saturated sodium
163 hydrogen carbonate solution. The reaction was extracted with ethyl acetate. The combined
164 organic phases were dried, filtered and concentrated under vacuum. The residue was
165 purified by chromatography on silica gel using a 0-20% DCM/MeOH to provide the desired
166 compound as a yellow solid (0.79g, 66%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.55 (dd,

167 1H), 7.91 (d, 1H), 7.78 (d, 1H), 7.40 (m, 2H), 7.17 (dd, 1H), 6.91 (d, 1H), 4.38 (t, 2H), 3.76

168 (s, 2H), 2.97 (d, 2H), 2.64 (t, 2H), 2.50 (m, 1H), 2.18 (t, 2H), 1.87 (d, 2H), 1.42 (m, 4H).

169 [ES+ MS] m/z 431.

170 **1-(2-(4-((3,4-Dichlorobenzyl)amino)piperidin-1-yl)ethyl)-1,5-naphthyridin-2(1H)-one**

171 **dihydrochloride (15).** To a solution of 1-(2-(4-((3,4-dichlorobenzyl)amino)piperidin-1-

172 yl)ethyl)-1,5-naphthyridin-2(1H)-one (940.6mg, 2.18mmol) in DCM (20 mL) was added

173 HCl (4M solution in 1,4-dioxan, 1.09 mL). After stirring 10 min, the obtained solid was

174 filtered and dried to give 1040mg (95%) of the title compound. ^{1}H NMR (400 MHz, d_6-

175 DMSO) δ ppm: 10.97-10.85 (m, 1H), 9.82-9.67 (m, 2H), 8.56 (d, 1H), 8.32-8.16 (m, 1H),

176 7.99-7.94 (m, 2H), 7.74-7.58 (m, 3H), 6.90 (d, 1H), 4.68-4.52 (m, 2H), 4.19 (s, 2H), 3.83-

177 3.66 (m, 1H), 3.21-3.04 (m, 2H), 2.40-1.90 (m, 4H). [ES+ MS] m/z 431. (M+H)+.

178 **1-(2-(4-((3,4-Dichlorobenzyl)amino)piperidin-1-yl)ethyl)-7-methoxy-1,5-naphthyridin-**

179 **2(1H)-one (16).** A suspension of 1-(2-(4-aminopiperidin-1-yl)ethyl)-7-methoxy-1,5-

180 naphthyridin-2(1H)-one dihydrochloride (0.189g, 0.46mmol) in DCM (6 mL) and MeOH

181 (0.6mL) was treated with triethylamine (0.21 mL, 1.51mmol) and stirred for 15min at rt

182 before the addition of 3,4-dichlorobenzaldehyde (commercial source) (0.076g, 0.43mmol)

183 in one portion. The mixture was stirred for 30min at rt and sodium triacetoxyborohydride

184 (0.9g, 4.11mmol) was added. The reaction was stirred overnight before addition of

185 saturated sodium hydrogen carbonate solution. The reaction was extracted with 20%

186 DCM/MeOH. The combined organic phases were dried, filtered and concentrated under

187 vacuum. The residue was purified by chromatography on silica gel using a 0-20%

188 DCM/MeOH to provide the desired compound as a yellow solid (82mg, 40%). ^{1}H NMR

189 (400 MHz, $CDCl_3$) δ ppm: 8.28 (d, 1H), 7.84 (d, 1H), 7.44 (s, 1H), 7.38 (d, 2H), 7.25 (bd,

190 1H), 7.16 (d, 1H), 6.73 (d, 1H), 4.38 (t, 2H), 3.98 (s, 3H), 3.77 (s, 2H), 2.99 (bd, 2H), 2.66
191 (t, 2H), 2.58-2.45 (m, 2H), 2.28-2.15 (m, 2H), 1.95 (d, 2H), 1.50-1.35 (m, 2H). [ES+ MS]
192 m/z 461 (M+H)+.
193

194 ***In vitro* assays**

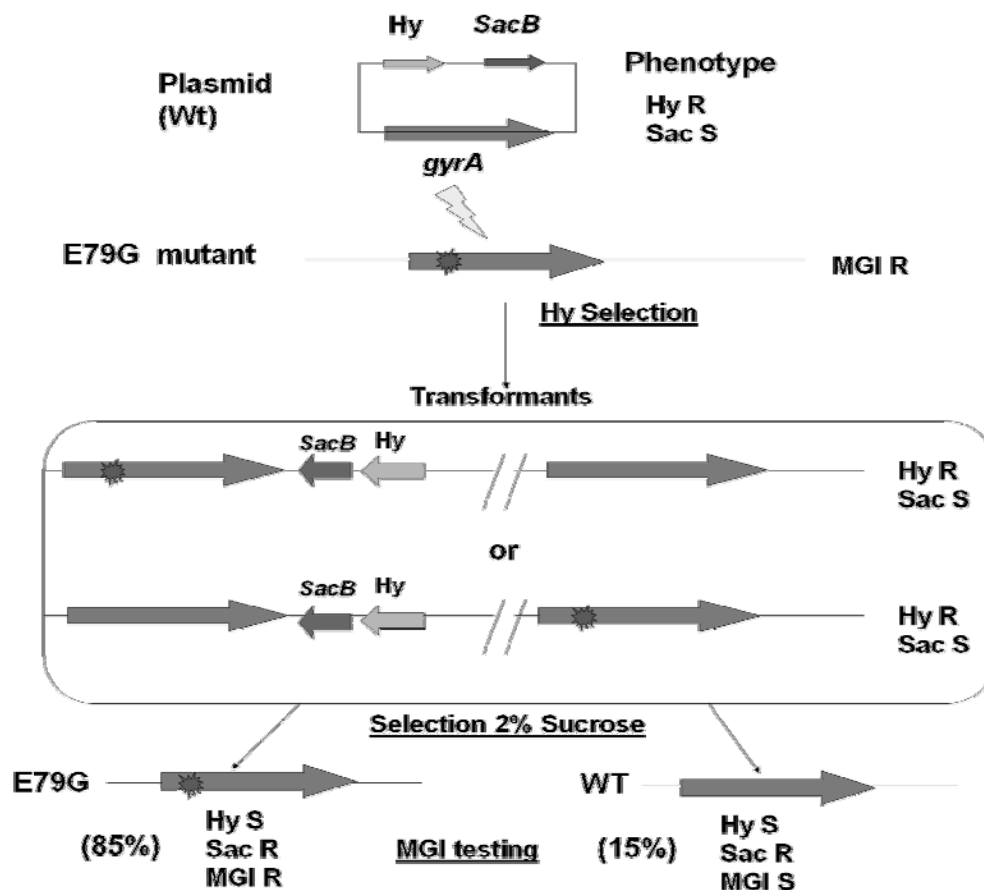
195 **Intracellular Viability assay**. THP1 cells are maintained in suspension with RPMI-1640
196 media containing 10% FBS, 1mM of Pyruvate, 2mM of L-Glutamine, and incubated at 37
197 ºC with 5% CO_2. Prior to day of infection, *M. tuberculosis* H37Rv was grown at 37ºC in
198 Middlebrock 7H9-ADC –Tween80 to mid-exponential phase and then diluted 1/100 in
199 200ml Middlebrock 7H9-ADC –Tween80 in a roller bottle and incubate at 37ºC until an
200 OD 600 nm between 0.6-1 at time of infection. 160 ml (5 x 50mL sterile tubes) of
201 mycobacterial cultures were pelleted at 3500 rpm for 10 min. The bacterial pellet of each
202 tube was aseptically dispersed by shaking with 10 glass beads during 60 seconds. Then, 6
203 mL of RPMI fresh media were added and it was left to sediment during 5 minutes.
204 Afterwards, 5 mL of the supernatant were taken carefully and the rest was discarded.
205 Finally, OD 600 nm of dispersed bacterial suspension was measured and the bacteria
206 number was calculated by using the following conversion: An OD of 0.1 = 10^8 CFU/mL.
207 Monocytes are grown to sub-confluence ($5x10^5$ cell/ml) and infected ON in a cell roller
208 bottle with a multiplicity of infection (MOI) of 10 for BCG or infected during 4h with a
209 MOI of 1 for H37Rv. Excess bacteria are removed by washing five times in RPMI media
210 (1500 rpm 5min.). Then, 100 ul/well (50.000 cells/well) of infected cells are dispensed in
211 96 well white plates. Afterwards 1:3 serial dilutions of antitubercular agents are added to
212 the cell plates. As DMSO damages cells, the percentage must be reduced under 0.5% by
213 diluting 1:400 in culture medium. Luminescence is measured after 5 days using the Steady-

214 Glo Promega kit into a Victor 1420 system. MIC90 values are calculated from the dose-

215 response curves by non-linear regression analysis.

216 **Cytotoxicity experiments.** Experimental procedures were used as described previously (1).

217 **Reversion of the mutations**.



218

219 **Fig. S1**. In the first homologous recombination, Hyg^R are selected as the ones which have

220 included the wild type gene. In the second homologous recombination, Hyg^S-$Sucrose^R$ are

221 selected as the ones that have lost HygR and SacB genes so that recombination has taken

222 place. Depending on whether this recombination takes place before or after the mutation,

223 we can obtain the resistant mutant or the wild type phenotype. 15% of the transformants

224 obtained had reverted to sensitivity.

225 **Microsomal fraction stability experimental procedure.** Experimental procedures were
226 used as described previously (2).
227 **Inhibition of *Mtb* DNA Gyrase by compounds.** The reactions were initiated by the
228 addition of 0.3 U of *Mtb* DNA Gyrase, incubated at 37 ºC for 60 min and then stopped
229 using 5 µL of 0.25% v/v bromophenol blue and 50% v/v glycerol. 25 µL (≈500 ng) of the
230 reaction was then electrophorosed in a 1% w/v agarose TAE (40 mM Tris (pH 8.3), 20 mM
231 acetic acid and 1 mM EDTA)) gel for 3 h at 75 V in TAE running buffer. The gel was
232 stained in TAE buffer containing Gel Red Nac Stain (0.7 µg/mL) for 1.5 h and then
233 visualized using a Gel Doc-It Imaging System (UVP Inc.). UVP Vision Works LS Software
234 was used to quantify the intensity and retention factor (Rf) of the supercoiled DNA band in
235 each lane and this data was fitted to a generic four-parameter logistical equation using
236 Grafit software.
237
238 ***In vivo* assays**
239 **General aspects and ethics Statement.** The compounds used in this study were prepared
240 as suspensions in 1% Methyl Cellulose. The antitubercular standards used in the efficacy
241 study were: Moxifloxacin (Sequoia Research Products Ltd) prepared as solution in 20%
242 Captisol(R)/water).
243 **Mice.** Specific Pathogen-free 6-8-week-old female C57BL/6j mice (18 – 20g) are obtained
244 from Harlan (Harlan Interfauna Iberica, Spain). The experiments were performed at
245 AAALAC-accredited GSK Laboratory Animal Science animal facilities in Tres Cantos
246 (Madrid, Spain). The mice were kept in air-conditioned facilities with fifteen air changes
247 per hour. Room temperature and relative humidity were 22 ± 3 ºC and 40- 70%,

248 respectively. The mice were accomodated in groups of up to five individuals in
249 Tecniplast® type IV cages with autoclaved dust free corncob bedding (Panlab, Barcelona,
250 Spain). The mice were maintained under a twelve hours light/dark period. Autoclaved tap
251 water and γ-irradiated pelleted diet were provided ad libitum.

252 All experiments were approved by the Diseases of the Developing World (DDW-GSK)
253 ethical committee. All animal studies were ethically reviewed and carried out in accordance
254 with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and
255 Treatment of Animals.

256 **Pharmacokinetic studies.** The experimental design has been previously described (2). In
257 brief, C57BL/6 female mice of 18-20 g weight were used (n=4 mice per time point).
258 Compound 1 was administered by oral gavage at 50 mg/kg single dose at a volume of 20
259 ml/kg to n=4 mice per time point in saline. Compound 2 was administered by intravenous
260 route at 10 mg/kg single dose at a volume of 10 ml/kg to n=4 mice per time point in saline
261 and by oral gavage at 50 mg/kg single dose at a volume of 20 ml/kg to n=4 mice per time
262 point in saline (pH=3.8). Compound 3 was administered by intravenous route at 5 mg/kg
263 single dose at a volume of 10 ml/kg to n=4 mice per time point in 20% encapsin/saline and
264 by oral gavage at 10 mg/kg single dose at a volume of 20 ml/kg to n=4 mice per time point
265 in water (pH=3.6). Peripheral total blood was the compartment chosen for the establishment
266 of compound concentrations: aliquots of 25 µl of blood were taken by cardiac puncture for
267 each mouse (euthanized by CO_2) at 10, 20, 30 minutes, 1, 1.5, 2, 3, 4 and 8 hours for
268 intravenous route and at 15, 30, 45 minutes, 1, 1.5, 2, 3, 4 and 8 hours for oral route (n=4
269 mice per time point).

270

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