

1 **Direct inhibitors of InhA with efficacy similar** 2 **or superior to isoniazid in novel drug** 3 **regimens for tuberculosis**

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18 Running Head: Direct InhA inhibitors improve TB regimens in mice

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26

27 **ABSTRACT**

28 **Isoniazid is an important first-line medicine to treat tuberculosis (TB). Isoniazid**
29 **resistance increases the risk of poor treatment outcomes and development of**
30 **multidrug resistance, and is driven primarily by mutations involving *katG*,**
31 **encoding the pro-drug activating enzyme, rather than its validated target, *InhA*.**
32 **The chemical tractability of *InhA* has fostered efforts to discover direct inhibitors**
33 **of *InhA* (DIs). During the past five years, successful target engagement and *in***
34 ***vivo* efficacy have been demonstrated by diverse DIs. In this study, we bridge the**
35 **gap in understanding the potential contribution of DIs to novel combination**
36 **regimens and demonstrate a clear distinction of DIs, like GSK693 and the newly**
37 **described GSK138, from isoniazid, based on activity against clinical isolates and**
38 **contribution to novel drug regimens. The results presented increase the**
39 **understanding of DI mechanism of action and provide further impetus to**
40 **continue exploiting *InhA* as a promising target for TB drug development.**

41 **INTRODUCTION**

42 Tuberculosis (TB) is a communicable disease caused by *Mycobacterium tuberculosis*
43 (*M.tb*). Globally, an estimated 10.6 million people developed TB in 2022, up from best
44 estimates of 10.3 million in 2021 and 10.0 million in 2020. Until the coronavirus (COVID-
45 19) pandemic, TB was the leading cause of death from a single infectious agent,
46 ranking above HIV/AIDS. The COVID-19 pandemic has had a negative impact on
47 access to TB diagnosis and treatment as well as the burden of TB disease. The
48 estimated number of deaths from TB increased between 2019 and 2021, reversing
49 years of decline. An estimated total of 1.3 million people died from TB in 2022 (including

50 167,000 people with HIV). The net reduction in the global number of deaths caused by
51 TB from 2015 to 2022 was 19%, far from the WHO End TB Strategy milestone of a 75%
52 reduction by 2025 (1, 2). With timely diagnosis and treatment with first-line drugs, most
53 people who develop TB are cured and onward transmission of infection is curtailed. The
54 currently recommended treatment for drug-susceptible pulmonary TB is a 6-month
55 regimen consisting of an *intensive phase* of 2 months with a 4-drug regimen of
56 isoniazid, rifampicin, pyrazinamide, and ethambutol, followed by a *continuation phase* of
57 four months with isoniazid and rifampicin.

58 Isoniazid was discovered in 1952 and has been widely used to treat TB ever since (3). It
59 is a prodrug that requires activation by the mycobacterial catalase-peroxidase enzyme
60 KatG to form the reactive isonicotinyl acyl radical (4), which then forms a covalent
61 adduct with the cofactor nicotinamide adenine dinucleotide (isoniazid-NAD adduct) (5).
62 This adduct is the inhibitor of the mycobacterial fatty acid synthase II (FAS-II)
63 component enoyl-acyl carrier protein reductase (InhA), which is required for the
64 synthesis of mycolic acids, a central component of the mycobacterial cell wall (6, 7).

65 Isoniazid is an important first-line TB drug. Baseline isoniazid resistance increases the
66 risk of poor treatment outcomes (e.g. treatment failure or relapse) and acquisition of
67 multidrug-resistant (MDR) TB. Based on evidence reviews indicating reduced efficacy of
68 the standard first-line drugs for the treatment of isoniazid-resistant TB (Hr-TB) (8-13),
69 the World Health Organization (WHO) issued a Supplement to its guidelines for the
70 treatment of drug-resistant TB in 2018, providing new recommendations for the
71 management of Hr-TB (14). Resistance to isoniazid is primarily caused by mutations in
72 the activating enzyme KatG or in the upstream promoter region of InhA or more rarely in

73 the InhA enzyme itself. Combinations of these mutations may also occur. By and large,
74 the most common mutations in Hr-TB strains are found in *katG* and confer “high-level”
75 resistance, even in the absence of an *inhA* mutation. In this situation, the inclusion of
76 isoniazid in the regimen, even at high doses, is unlikely to increase its effectiveness
77 (although this question is currently under investigation: *ClinicalTrials.gov Identifier:*
78 *NCT01936831*). On the other hand, mutations in the *inhA* promoter or in the *inhA* gene
79 are generally associated with lower-level resistance than *katG* mutations, and higher
80 doses of isoniazid (10-15 mg/kg/day) may result in bactericidal activity against such
81 *inhA* mutants similar to that observed with standard isoniazid doses (4-6 mg/kg/day)
82 against fully susceptible strains (4, 15-17).

83 The opportunity to overcome the high rate of clinical resistance to isoniazid due to *katG*
84 mutations, together with the biological relevance of InhA (target validated clinically by
85 isoniazid and ethionamide) and its chemical tractability, (18) has fostered efforts to
86 discover direct inhibitors of InhA (DIIs). During the last five years, three structurally
87 different molecules have demonstrated *in vivo* efficacy in murine TB
88 models upon oral administration: **NITD-916** (19), **GSK2505693A (GSK693)** (20) and
89 **AN12855** (21).

90 **RESULTS**

91 To our knowledge, **GSK693** was the first DII compound to demonstrate *in vivo* efficacy
92 comparable to that of isoniazid (20). More recently, Xia and coauthors reported the
93 discovery of a direct, cofactor-independent inhibitor of InhA, AN12855, which showed
94 good efficacy in acute and chronic murine TB models that was also comparable to

95 isoniazid (20). The high preliminary human dose prediction of **GSK693** hampered its
96 further development as a lead compound. Within the same thiadiazole-based series,
97 **GSK3081138A (GSK138)**, a structurally very similar and slightly more lipophilic
98 compound, was selected as a back-up compound based on its balanced profile of
99 physicochemical properties, *in vitro* potency, *in vivo* pharmacokinetics (PK), and safety
100 (Table 1).

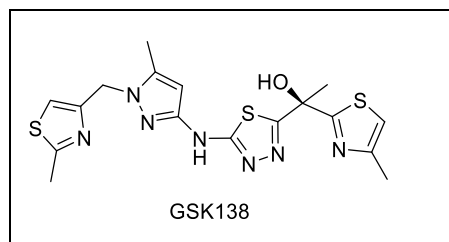
101 **GSK138** is a medium molecular weight compound with a chrom logD at pH 7.4 of 3.38.
102 The measured solubility in Fasted State Simulated Intestinal Fluid (FaSSIF) was high
103 (Table 1). The permeability of **GSK138** predicted from Madin-Darby Canine Kidney
104 (MDCK) cells was also high (Table 1). The efflux ratio determined by assays with and
105 without incubation with a potent P-glycoprotein (P-gp) inhibitor indicated that it is a P-gp
106 substrate.

107 **GSK138** inhibited recombinant InhA with an IC₅₀ of 0.04 µM. The MIC was 1 µM against
108 *M. tuberculosis* H37Rv and **GSK138** retained its activity against intracellular bacteria
109 growing inside THP-1-derived macrophages *in vitro* (MIC 0.9 µM). Additionally, it
110 showed no effect up to the highest concentration tested (200 µM) in the Cell Health
111 assay (measuring membrane, nuclear, and mitochondrial damage). The preliminary
112 toxicological profile showed an overall clean *in vitro* safety profile.

113 To assess the susceptibility of **GSK138** to P450-mediated phase I metabolism,
114 metabolic stability was determined during incubation in CD1 mouse, Sprague Dawley
115 rat, beagle dog, and human liver microsomes. **GSK138** exhibited moderate *in vitro*
116 clearance in liver microsomes from the pre-clinical species, and low *in vitro* clearance in
117 humans.

118 To determine the pharmacokinetic parameters, **GSK138** was administered
 119 intravenously (formulation: 5%DMSO/20%Encapsin in saline solution) as a single bolus
 120 dose in C57BL/6 mice at a target dose of 1 mg/Kg. All pharmacokinetic parameters
 121 were determined in whole blood (Table 1). A moderate clearance and a moderate
 122 volume of distribution were observed.

123
 124 TABLE 1. Structure and properties of the optimized lead **GSK138**. The lead was assessed for
 125 activity against *M. tuberculosis* H37Rv both intracellularly and extracellularly. The
 126 physicochemical and ADMET properties were determined as well.



Physicochemical properties	MW	433
	clogP/Chrom logD	1.2/3.39
	Permeability Papp (MDCK-MDR1)	374 nm/s
	Solubility FaSSIF (pH 6.5)	140-320 μ M
Activity profile	InhA IC ₅₀ *	0.04 μ M \pm 0.01
	<i>Mtb</i> MIC	1 μ M
	<i>Mtb</i> intracell MIC*	0.9 μ M \pm 0.1
Cytotoxicity profile	HepG2 Cytotoxicity Tox ₅₀	>100 μ M
	Cell Health (nuclear size, mitochondrial membrane potential and plasma membrane permeability)	>199.5 μ M
Genetic toxicity assessment	Ames test	Negative
Cardiovascular profile	hERG Qpatch IC ₅₀	>30 μ M
Microsomal stability assessment	<i>In vitro</i> Cli mouse/rat/dog/human	4.4 /3.5/1.7/0.3 mL/min/g tissue
<i>In vivo</i> pharmacokinetic profile	<i>In vivo</i> Cl mouse (1 mg/Kg iv)*	77.6 \pm 16.8 mL/min/Kg
	<i>In vivo</i> Vss mouse (1 mg/Kg iv)*	2.6 \pm 0.3 L/Kg

127 *mean \pm Standard Deviation.

128

129 The minimum concentrations of **GSK693** and **GSK138** that inhibit 90% of isolates
 130 tested (MIC₉₀) were determined against a set of drug-susceptible, multidrug-resistant
 131 (MDR) and extensively drug-resistant (XDR) *M.tb* clinical isolates. Both **GSK693** and
 132 **GSK138** retained activity against these clinical isolates (**GSK693** MIC₉₀ = 1.87 μM;
 133 **GSK138** MIC₉₀ = 3.75 μM), similar to the MICs against strain H37Rv in the same assay
 134 (Table 2). As expected for DIs, the thiadiazole compounds have KatG-independent
 135 activity. No change in MIC was observed against isoniazid-resistant clinical isolates
 136 carrying mutations in *katG* S315T. Clinical isolates carrying an *inhA* C-15T mutation
 137 have increased InhA production which confers low-level resistance to isoniazid. Among
 138 eight clinical isolates with the *inhA* C-15T mutation, three showed low-level resistance to
 139 the thiadiazoles (i.e., MICs for both DIs ≥4 times the MIC against strain H37Rv).
 140 Thiadiazoles remained equally effective among the rest of the sensitive, MDR and XDR
 141 *M.tb* clinical isolates tested.

142
 143 TABLE 2. Activity of **GSK693** and **GSK138** against resistant *M.tb* clinical isolates obtained from
 144 Vall d’Hebron Hospital, Barcelona. Resistance pattern: **H = isoniazid (katG S315T mutation:G,**
 145 **inhA promoter C-15T mutation:P)**, R = rifampicin, Z = pyrazinamide, M = moxifloxacin, T =
 146 ethionamide, S = streptomycin, E = ethambutol, K = kanamycin, A = amikacin, Cm =
 147 capreomycin, O = ofloxacin, Cp = ciprofloxacin, and Pas = para-aminosalicylic acid

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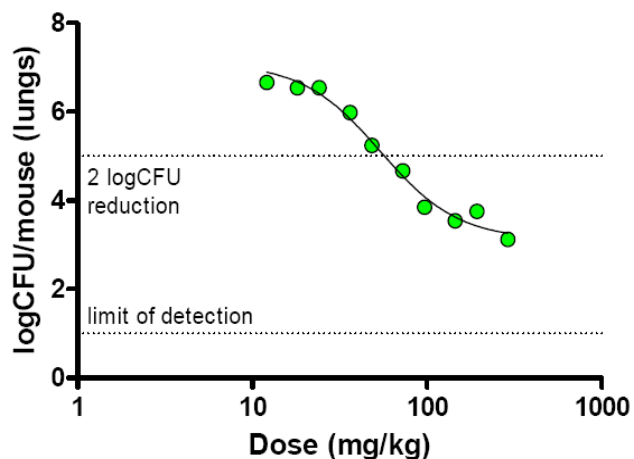
Strain #	Strain ID	Resistance	693 MIC (μM)	138 MIC (μM)
1	13243	MOCp	<0.23	<0.23
2	12569	OCp	<0.23	<0.23
3	9685A	H(G)RESZCpT	<0.23	<0.23
4	7788	RZCmK	<0.23	0.23
5	14388	H(G)RESZCmAT	<0.23	0.47
6	13026	H(G)RESCmKCpO	<0.23	0.47
7	13214	H(P)SPas	<0.23	0.47
8	12733	H(G)EZOTPas	<0.23	0.47
9	7957	KCp	<0.23	0.47

10	10841	H(G)RESZMO	<0.23	0.47
11	10071	H(G)RESZKCp	<0.23	0.47
12	11881	H(G)RESZCmKCp	<0.23	0.47
13	8059	Cp	<0.23	0.47
14	14294	ZMO	0.23	0.23
15	11341	H(G)REOPas	0.23	0.47
16	13830	H(G)RESZCmKMO	0.47	0.47
17	14379	H(G)RESZMO	0.47	0.47
18	14883	H(G)RESZCp	0.47	0.94
19	13222	SCmMCpO	0.47	0.94
20	10492	RSCp	0.47	0.94
21	11586	H(G)RESKCp	0.94	1.875
22	11347	H(P)RECmKO	0.94	1.875
23	10027	H(G)RESZCmKCp	0.94	1.875
24	7786	H(P)RESCmKCp	1.875	1.875
25	10190	H(P)REZCp	1.875	1.875
26	7543	H(P)RESZCmKCp	1.875	1.875
27	11366	H(GP)RESCmKO	1.875	3.75
28	11348	H(P)RESOTPas	3.75	7.5
29	13229	H(P)RZMCpOT	7.5	>15
30	H37Rv	Susceptible (control)	<0.23	0.94
31	14639	Susceptible (control)	0.47	0.47

149

150 Based on **GSK138**'s overall profile, the therapeutic efficacy of **GSK138** against *M.tb* in

151 an acute murine model of intratracheal infection was determined (see FIG 1).

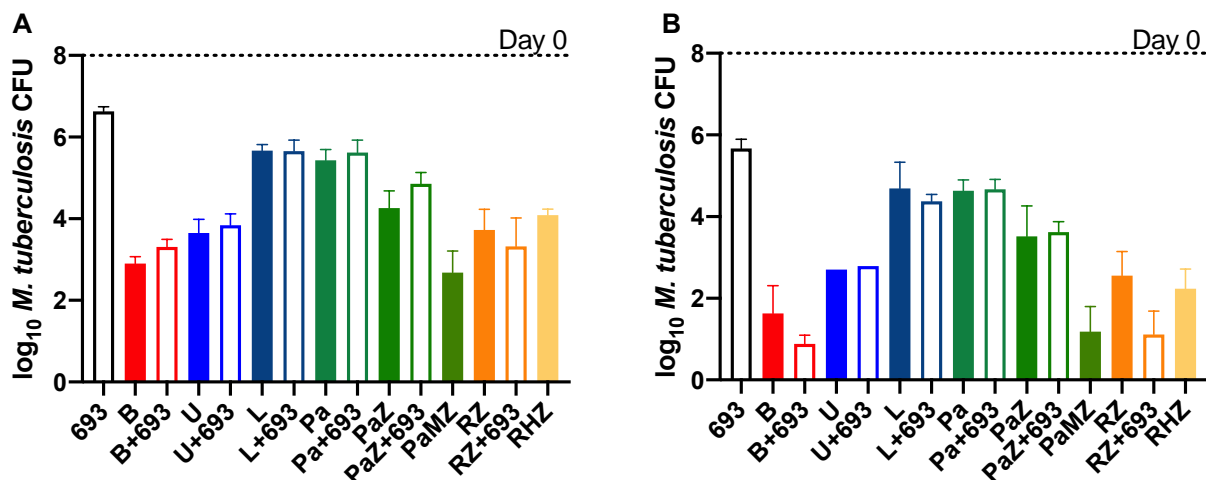


152
153 FIG 1. Dose-response relationship for **GSK138** in an acute mouse infection model of TB. Each
154 point represents data from an individual mouse that received **GSK138** administered orally once
155 daily for 8 days.

156
157 This acute infection model measures antitubercular activity on fast-growing bacteria
158 (22). Treatment is administered for 8 consecutive days, starting 1 day after infection.
159 Because the bacterial load reduction previously observed with **GSK693** was similar in
160 both acute and chronic murine TB models (20), we performed a full dose-response
161 study only in the acute model to characterize the compound and estimate the optimal
162 dose for future combination studies. **GSK138** induced a net killing of the bacteria at the
163 highest doses. The ED₉₉ (the dose producing a 2-log₁₀ reduction in colony-forming units
164 [CFUs] compared to untreated control mice) for **GSK138** was 57 mg/Kg (95%
165 confidence interval [CI]: 50-67 mg/Kg) and the dose of **GSK138** at which the 90% of the
166 maximum bactericidal effect was achieved (ED_{max}) was 167 mg/Kg (95%CI: 125->290
167 mg/Kg). The whole blood area under the concentration-time curve over 24 hours post-
168 dose (AUC_{0-24h}) at steady-state associated with this ED_{max} (AUC_{EDmax}) was 68,544

169 ng*h/mL. Comparison with previous data suggests that **GSK138** is as efficacious as
170 **GSK693** at a lower exposure, and therefore **GSK138** has the potential for a lower dose
171 prediction in humans.

172 Ultimately, any antitubercular drug must be used in combination with other anti-
173 tubercular drugs to treat active TB. The success of any new regimen will depend on the
174 properties of these drugs and how they work in combination. **GSK693** and **GSK138**
175 showed suitable profiles to justify investigation of the efficacy of these DIIIs in
176 combination with other drugs in animal models. Firstly, **GSK693** was selected as a tool
177 compound to learn about the chemical series and its interactions with potential
178 companion drugs. Experiment 1 was performed in a well-established high-dose aerosol
179 infection model (23) with the following objectives: 1) to evaluate its ability to replace
180 isoniazid (H) in combination with rifampicin (R) and pyrazinamide (Z) in the core first-
181 line regimen, 2) to evaluate its ability to replace moxifloxacin (M) in combination with
182 pretomanid (Pa) and pyrazinamide in the novel PaMZ regimen, and 3) to evaluate its
183 contribution to the bactericidal activity of 2-drug combinations including bedaquiline (B),
184 sutezolid (U), linezolid (L) and pretomanid (Figure 2).



185
186 FIG 2. Mean (\pm SD) lung CFU counts at D0 and after 4 (A) or 8 (B) weeks of treatment in Expt
187 1. In combination with RZ, but not with other drugs, **GSK693** showed significantly enhanced
188 antibacterial activity at week 8 (B) but not at week 4 (A). Open bars show lung CFU counts with
189 the addition of **GSK693** to drugs shown in solid bars. Drug doses: R = rifampicin 10 mg/Kg, Z =
190 pyrazinamide 150 mg/Kg, H = isoniazid 10 mg/Kg, 693 = **GSK693** 300 mg/Kg, Pa = pretomanid
191 50 mg/Kg, M = moxifloxacin 100 mg/Kg, B = bedaquiline 25 mg/Kg, L = linezolid 100 mg/Kg, U
192 = sutezolid 50 mg/Kg.

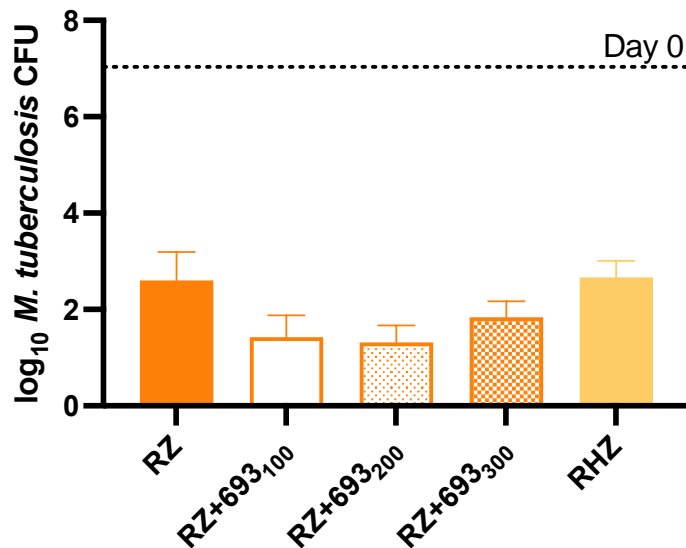
193 In this model, in which untreated mice routinely succumb to infection with lung CFU
194 counts above 8 \log_{10} within the first 3-4 weeks after infection, **GSK693** (300 mg/Kg)
195 reduced the lung CFU counts by 1.34 and 2.33 \log_{10} after 4 and 8 weeks of treatment,
196 respectively. This bactericidal effect approached that of linezolid or pretomanid. No
197 additive effect was observed when **GSK693** was combined with sutezolid, linezolid or
198 pretomanid, nor was it as effective as moxifloxacin in combination with pretomanid and
199 pyrazinamide. However, the combination of **GSK693** with rifampicin and pyrazinamide
200 (RZ) was significantly more active than RZ alone or in combination with isoniazid after 8
201 weeks of treatment ($p < 0.05$). Notably, the combination of **GSK693** with bedaquiline also

202 resulted in greater activity after 8 weeks of treatment ($p=0.08$) than that observed with
203 bedaquiline alone. This additive effect of **GSK693** was attributable to its prevention of
204 selection of bedaquiline-resistant mutants, as emergence of bedaquiline resistance was
205 observed in 2 of the 4 mice treated with bedaquiline alone for 8 weeks, consistent with
206 previous results (24). Excluding these 2 mice from the analysis revealed no difference
207 between treatment with bedaquiline alone and bedaquiline plus **GSK693**.

208 The promising result observed with RZ+**GSK693** (in Exp. 1) prompted a follow-up
209 experiment to confirm the additive effect of **GSK693**, evaluate the dose-response
210 relationship for **GSK693** and explore potential drug-drug interactions in the RZ+**GSK693**
211 combination.

212 As observed in Experiment 1, the addition of **GSK693**, but not isoniazid, significantly
213 increased the activity of the RZ combination in Experiment 2 (Figure 3).

214



215
216 FIG 3. Mean (\pm SD) lung CFU counts at D0 and after 8 weeks of treatment in Experiment 2.

217 693 = **GSK693** significantly enhanced, in a non-dose-dependent manner, the activity of the RZ

218 (rifampicin, 10 mg/Kg, plus pyrazinamide 150 mg/Kg) combination. Isoniazid (10 mg/Kg) did not
219 enhance the activity of the combination. **GSK693** dose (in mg/Kg) is indicated in subscripts.

220
221 The magnitude of the additive effect was also similar between experiments. The
222 addition of **GSK693** at 300 mg/Kg to RZ reduced the lung CFU counts by an additional
223 1.44 log in Experiment 1, as compared to a reduction of 0.76 log ($p < 0.05$ vs RZ) in
224 Experiment 2. Remarkably, however, greater reductions of 1.17 and 1.28 log ($p < 0.01$
225 and 0.001 vs RZ, respectively) were observed when **GSK693** was used at 100 and 200
226 mg/Kg, respectively, in Experiment 2.

227 Although the sparse sampling prevented a formal assessment of the PK profile, the
228 apparent lack of **GSK693** dose response was not explained by the **GSK693** exposures
229 at the 100, 200 and 300 mg/Kg doses (Table 3).

230 TABLE 3. Data obtained from monocompartmental model of sparse plasma sampling
231 concentrations in the combination study. A blood/plasma ratio of 1.79 was used to transform the
232 plasma parameters to blood values.

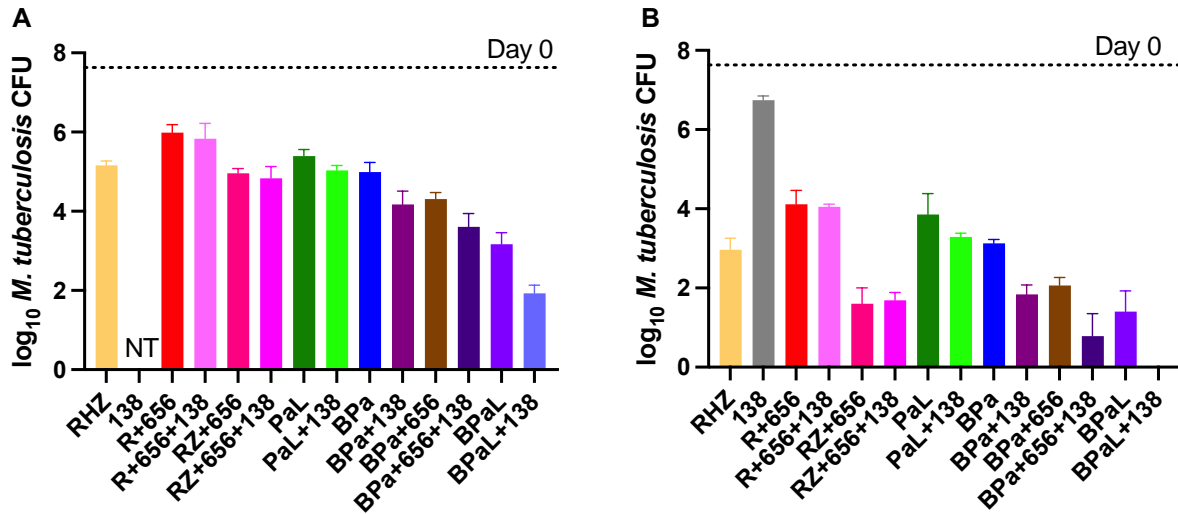
GSK693 Dose	Plasma AUC_{0-24h} (ng·h/mL)	Blood AUC_{0-24h} (ng·h/mL)
100 mg/Kg	12,867	23,032
200 mg/Kg	27,337	48,933
300 mg/Kg	64,866	116,110

233
234 Interestingly, the observed effect of **GSK693** in combination was achieved at a lower
235 exposure than that needed to achieve the maximum effect in monotherapy in the acute
236 infection model (110,200 ng·h/mL). Based upon the potential for drug-drug interactions,

237 rifampicin was administered 1 hour prior to other drugs (25). Plasma exposure of
238 rifampicin ($AUC_{0-24h} = 68,544$ ng·h/mL) when co-administered with pyrazinamide
239 showed no evidence of a higher exposure that could explain the increase in the efficacy
240 of the combination when compared to prior data for rifampicin when co-administered
241 with pyrazinamide ($AUC_{0-24h} = 160,600$ ng·h/mL) (25) or as monotherapy at 10 mg/kg
242 ($AUC_{0-24h} = 87,200$ to $142,100$ ng·h/mL) (26).

243 The result from the combination of **GSK693** with RZ proved to be superior to the first-
244 line treatment (RHZ). This result encouraged further combination experiments, now with
245 **GSK138**.

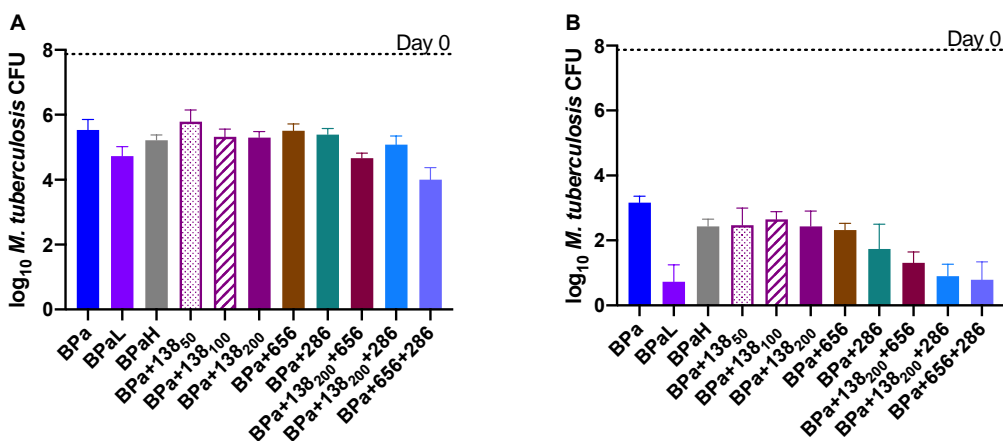
246 A major objective of Experiment 3 was to determine the effect of adding **GSK138** to the
247 novel regimen of bedaquiline, pretomanid, and linezolid (BPaL) recently approved for
248 treatment of XDR and treatment-intolerant or non-responsive MDR TB and the effect of
249 substituting **GSK138** for either bedaquiline or linezolid. The experiment also included
250 the novel LeuRS inhibitor GSK3036656 (GSK656) (27, 28) that is now in phase 2
251 clinical trials. The objectives of this experiment were the following: 1) to evaluate the
252 contribution of **GSK138** to the efficacy of 3- and 4-drug combinations based on the BPa
253 backbone, and 2) to evaluate the effect of adding **GSK138** to the combination of
254 rifampicin plus GSK656, with or without pyrazinamide (Figure 4).



255 FIG 4. The direct InhA inhibitor **GSK138** enhanced the activity of the BPa, BPAL and
 256 BPa+GSK656 combinations after 4 weeks (A) or 8 weeks (B) of treatment. After 8 weeks of
 257 treatment, the BPAL+**GSK138** regimen rendered mouse lungs culture negative. Data are
 258 presented as mean (\pm SD) lung CFU counts. R = rifampicin 10 mg/Kg, Z = pyrazinamide 150
 259 mg/Kg, H = isoniazid 10 mg/Kg, 138 = **GSK138** 200 mg/Kg, 656 = GSK656 (sulfate salt) 10
 260 mg/Kg, Pa = pretomanid 50 mg/Kg, B = bedaquiline 25 mg/Kg, L = linezolid 100 mg/Kg. NT =
 261 not tested.

262 The addition of **GSK138** to BPAL, its BPa backbone, or the novel BPa+GSK656
 263 regimen significantly increased the activity of each combination after 4 weeks ($p < 0.01$)
 264 and after 8 weeks ($p < 0.0001$) of treatment. Indeed, the 4-drug combination of BPAL plus
 265 **GSK138** was the only regimen tested to render all mice culture-negative after 8 weeks
 266 of treatment. After 8 weeks of treatment, the activity of the 3- and 4-drug regimens
 267 containing BPa plus **GSK138**, with or without GSK656, were statistically
 268 indistinguishable from that of BPAL and significantly superior to the first-line RHZ
 269 regimen ($p < 0.0001$). The addition of **GSK138** did not significantly increase the activity of
 270 PaL, R+GSK656, or RZ+GSK656.

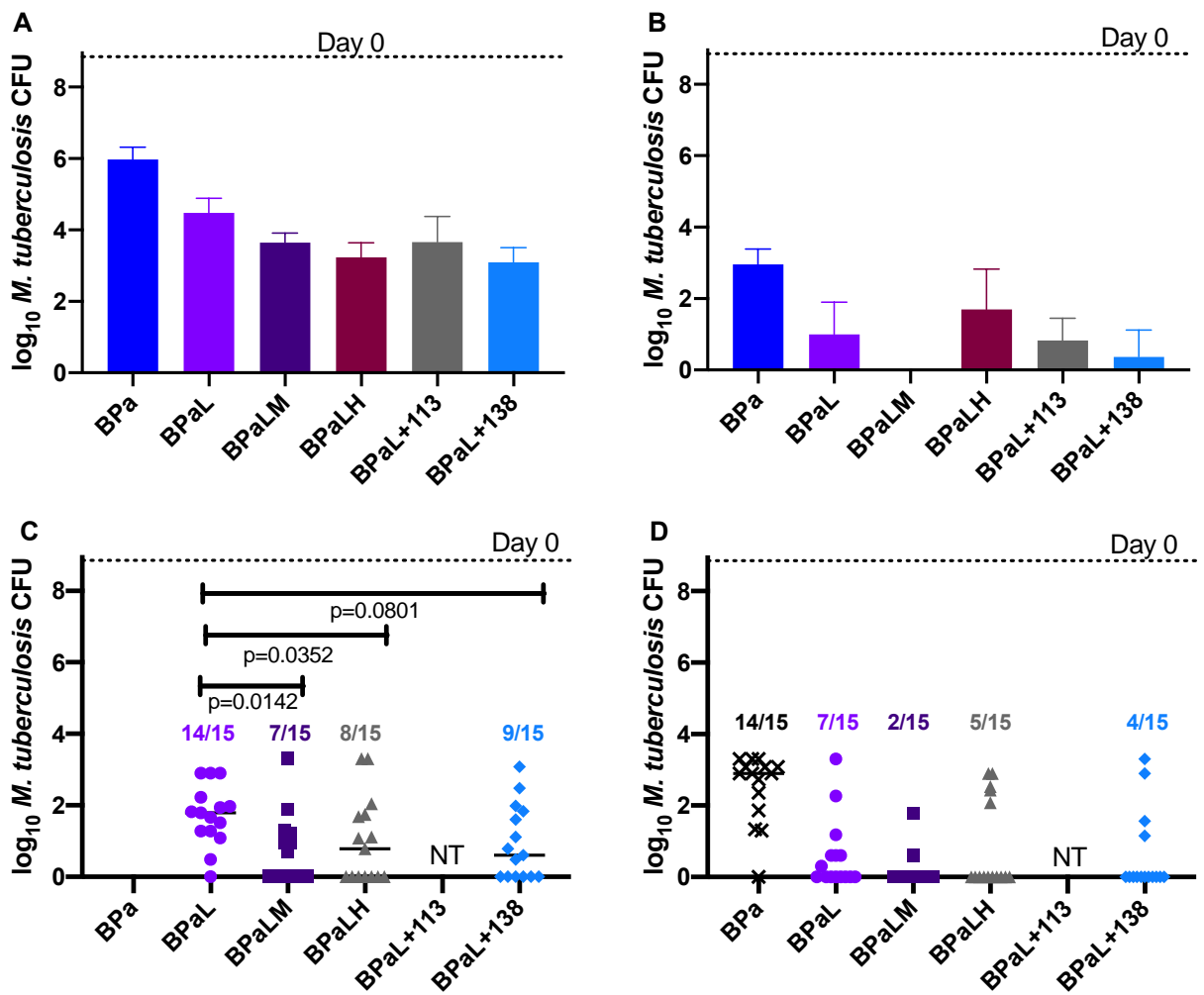
271 Experiment 4 (Figure 5) was performed to confirm the contribution of **GSK138** to the
272 BPa backbone, this time including a range of **GSK138** doses. The experiment also
273 included isoniazid as a comparator and combinations with GSK656 and the novel
274 cholesterol-dependent inhibitor GSK2556286 (GSK286) (29) which is currently being
275 investigated in a first time in human (FTIH) study to evaluate its safety, tolerability, and
276 pharmacokinetics (NCT04472897).



277
278 FIGURE 5. **GSK138** significantly enhanced the activity of BPa and BPa-based regimens at 4
279 weeks (A) or 8 weeks (B), particularly in combination with GSK286. In combination with
280 GSK656 or GSK286, the 200 mg/Kg dose of **GSK138** was used. Data are presented as mean
281 (\pm SD) lung CFU counts B = bedaquiline, 25 mg/Kg; Pa = pretomanid, 100 mg/Kg, L = linezolid,
282 100 mg/Kg, H = isoniazid 10 mg/Kg, 286 = GSK286, 50 mg/Kg, 656 = GSK656 (hydrochloride
283 salt), 10 mg/Kg. **GSK138** dose (in mg/Kg) is indicated in subscripts.

284
285 The results reaffirmed the additive effects of **GSK138** when added to BPa for 8 weeks
286 of treatment, and similar results were observed with the addition of isoniazid or GSK656
287 to BPa. No dose-response of **GSK138** was evident after 8 weeks; and unlike in

288 Experiment 3, BPa plus **GSK138** was less effective than BPaL ($p < 0.01$ after 4 and 8
 289 weeks). However, as observed in Experiment 3, the additive 4-drug combination of
 290 BPa+GSK656 with **GSK138** was statistically indistinguishable from BPaL, as was the
 291 combination of BPa+GSK286 with **GSK138**. These 4-drug combinations of
 292 BPa+**GSK138** plus either GSK656 or GSK286 also had bactericidal activity similar to
 293 BPa+GSK656+GSK286 after 8 weeks of treatment.



294
 295 FIG 6. The addition of an InhA inhibitor or moxifloxacin enhanced the bactericidal and sterilizing
 296 activity of the BPaL regimen. After both 4 weeks (A) and 8 weeks (B), the addition of

297 moxifloxacin, isoniazid, NITD-113, or **GSK138** to BPaL enhanced the bactericidal activity
298 compared to BPaL alone (with the exception of isoniazid at week 8). Data are presented as
299 mean (\pm SD) lung CFU counts. The proportion of mice relapsing after 8 weeks of treatment,
300 followed by 12 weeks of no treatment (C) was statistically significantly lower in the presence of
301 either moxifloxacin or isoniazid and approached statistical significance with **GSK138**. There
302 were fewer relapses after 12 weeks of treatment and 12 weeks of follow-up (D) with the addition
303 of a fourth drug, although these differences were not statistically significant. The proportions of
304 mice relapsing are indicated above the symbols for lung CFU counts. B = bedaquiline, 25
305 mg/Kg, Pa = pretomanid, 100 mg/Kg, L = linezolid, 100 mg/Kg, M = moxifloxacin 100 mg/Kg, H
306 = isoniazid 10 mg/Kg, NITD-113 = prodrug for NITD-916 (see Introduction) 150 mg/Kg, 138 =
307 **GSK138**200 mg/Kg. NT = not tested.

308

309 Given the superior bactericidal activity of the BPaL plus **GSK138** regimen compared to
310 BPaL alone in Experiment 3, Experiment 5 was performed to determine whether
311 addition of GSK138 to BPaL could shorten the duration of treatment needed to prevent
312 relapse (Figure 6). Comparator regimens included BPaL plus one of the following:
313 isoniazid, NITD-113 (prodrug for NITD-916, a previously reported DII based on a
314 different scaffold than **GSK138**) (19) and moxifloxacin (M). As observed in Experiment
315 3, the addition of **GSK138** at a dose of 200 mg/Kg significantly increased the
316 bactericidal activity of BPaL after 4 weeks of treatment ($p < 0.01$), as did isoniazid
317 ($p < 0.01$), while there was a trend towards enhanced activity with NITD-113 ($p = 0.11$)
318 and moxifloxacin ($p = 0.10$). BPaL+**GSK138** resulted in fewer culture-positive mice and a
319 lower mean CFU count after 8 weeks compared to BPaL and BPaL plus other InhA
320 inhibitors, although these differences were not statistically significant. Only the BPaLM

321 regimen rendered all mice culture-negative at this time point. Similarly, the addition of
322 **GSK138**, isoniazid or moxifloxacin to BPaL each reduced the proportion of mice
323 relapsing after 8 and 12 weeks of treatment compared to BPaL alone, although the
324 differences were statistically significant only after 8 weeks of treatment with moxifloxacin
325 or isoniazid, as shown in Figure 6C.

326 **DISCUSSION**

327 The thiadiazole-based DII (namely **GSK693**) proved capable of replacing isoniazid in
328 the first-line regimen. In fact, the bactericidal activity of the regimen increased with this
329 substitution and **GSK693** increased the activity of the rifampicin-pyrazinamide
330 combination. These results suggest that use of a DII instead of isoniazid could also
331 increase the sterilizing activity of the regimen. The superior activity of the DII in this
332 regimen may be the result of superior killing of phenotypically INH-tolerant persisters
333 that have relatively lower *katG* expression, whether stochastically or in response to
334 stress (30). Further development of thiadiazole DII could yield superior first-line
335 regimens containing rifamycins and pyrazinamide.

336 The thiadiazole-based DII (namely **GSK138**) also proved capable of increasing the
337 bactericidal and sterilizing activity of BPa-based regimens. BPaL is an effective 6-month
338 all-oral regimen for XDR-TB and difficult-to-treat MDR-TB cases (31, 32). The improved
339 efficacy observed with the addition of **GSK138** suggests that this or another DII could
340 further improve the BPaL regimen by increasing the overall cure rate, shortening
341 treatment duration and/or reducing the emergence of drug resistance. Considering the
342 strong overall activity of BPa+**GSK693** and **GSK693**'s ability to prevent selection of
343 bedaquiline-resistant mutants, the DII of this class could also reduce the need for

344 linezolid, the most toxic component of the BPaL regimen allowing lower doses and/or
345 shorter durations of linezolid.

346 The use of thiadiazole DIs alone or in combination with GSK656 to replace linezolid
347 entirely, if proven safe, could enable the use of BPa-based regimens as alternative,
348 more universally active, first-line regimens that would be less affected by isoniazid
349 monoresistance or MDR.

350 Although not the focus of this report, we observed that the addition of moxifloxacin to
351 BPaL improved the bactericidal and sterilizing activity of the regimen. BPaL and BPaLM
352 were studied in the TB-PRACTECAL trial (ClinicalTrials.gov Identifier: NCT02589782).
353 Our results, which were obtained before the start of the trial, predicted superior efficacy
354 of the 4-drug combination. Indeed, a higher rate of sputum culture conversion at 8
355 weeks was observed in TB-PRACTECAL participants receiving treatment with BPaLM
356 vs. BPaL (77% vs. 46%) (33).

357 This research adds to the limited knowledge of the activity of direct InhA inhibitors in
358 combination with new and existing TB drugs. The results suggest that a direct InhA
359 inhibitor (e.g., **GSK138** and **GSK693**) could be a promising partner in novel drug
360 regimens, enhancing their efficacy and/or preventing the selection of bedaquiline-
361 resistant mutants. These findings increase our understanding of the mechanism of
362 action of direct inhibitors of InhA and provide further impetus to continue exploiting InhA
363 as a promising target for TB drug development.

364 **MATERIALS AND METHODS**

365 The human biological samples were sourced ethically, and their research use was in
366 accord with the terms of the informed consents under an IRB/EC approved protocol. All

367 animal studies performed by GSK were ethically reviewed and carried out in
368 accordance with European Directive 2010/63/EEC and the GSK Policy on the Care,
369 Welfare and Treatment of Animals. All animal studies performed at Johns Hopkins
370 University (JHU) were conducted in accordance with the GSK Policy on the Care,
371 Welfare and Treatment of Laboratory Animals and were approved by the Institutional
372 Animal Care and Use Committee of JHU.

373 **Chemistry.**

374 A micromilling method was applied to **GSK138** for particle size reduction in order to
375 obtain a micronized GSK138 that was used in *in vivo* experiments. The Mixer Mill MM
376 301 (Retsch) was used at a frequency of 20 Hz for four cycles of 5 minutes.

377 NMR spectra were recorded on an Agilent Inova 600 MHz spectrometer equipped with
378 a 5 mm Triple Resonance Gradient Probe IDTG600-5 (experiments run under software
379 version vnmr3.2 Revision A). Measurements were made at room temperature in DMSO-
380 d_6 solvent. The chemical shift (δ) values are expressed in parts per million (ppm) and
381 coupling constants are in Hertz (Hz). The chemical shifts (δ) were given relative to the
382 residual ^1H and ^{13}C signals of the solvent peak as an internal standard: in ^1H NMR (600
383 MHz) δ 2.49 ppm (quin, $\text{C}_2\text{D}_5\text{HOS}$) for DMSO- d_6 ; in ^{13}C NMR (150 MHz) δ 40.07 ppm
384 (sept) for DMSO- d_6 . Legend: s = singlet, d = doublet, sept = septet, br = broad signal.

385 LC-MS purity data were collected using a Waters Acquity UPLC instrument coupled with
386 Waters Acquity single quadrupole mass and photodiode array detectors. High-resolution
387 MS (HRMS) was performed on a QSTAR Elite System mass spectrometer. ^1H NMR
388 (600 MHz, DMSO- d_6): δ 10.67 (s, 1H, NH), 7.26 (s, 1H, OH), 7.18 (s, 1H), 7.09 (s, 1H),
389 5.79 (s, 1H), 5.16 (s, 2H), 2.59 (s, 3H), 2.29 (s, 3H), 2.26 (s, 3H), 1.97 (s, 3H). ^{13}C NMR

390 (150 MHz, DMSO- d_6): δ 175.96, 166.46, 166.29, 164.69, 152.55, 152.26, 147.61,
391 140.88, 116.47, 115.38, 94.56, 74.68, 48.82, 28.98, 19.29, 17.51, 11.53. HRMS (ESI)
392 m/z : calcd for $C_{17}H_{19}N_7OS_3$ [M + H]⁺, 434.0891; found, 434.0889.

393 **Permeability studies.** Studies were performed as described by Polli et al. (34), with
394 minor modifications. GF120918 was used as the inhibitor of P-gp. Apical-to-basolateral
395 (A-to-B) and basolateral-to-apical (B-to-A) transport were studied across MDR1-MDCKII
396 cell monolayers in the absence and presence of the P-gp inhibitor GF120918, and the
397 Papp (intrinsic apparent permeability) was estimated in both directions with or without
398 inhibitor.

399 **Solubility studies.** Solubility assays were performed using a miniaturized shake flask
400 method. 10 mM stock solutions of each test compound were used to prepare calibration
401 standards (10-220 μ M) in DMSO, and to spike (1:50) duplicate aqueous samples of
402 FaSSIF (simulating fasting state biorelevant media, pH 6.5), with a final DMSO
403 concentration of 2%. After shaking for 2 hours at 25 °C, the solutions were filtered and
404 analysed by means of HPLC-DAD (Agilent 1200 Rapid Resolution HPLC with a diode
405 array detector). Best fit calibration curves were constructed using the calibration
406 standards, which were used to determine the aqueous samples solubility (35).

407 **Bacterial strains.** *M. tuberculosis* H37Rv was mouse-passaged, frozen in aliquots and
408 sub-cultured in Middlebrook 7H9 broth with 10% oleic acid-albumin-dextrose-catalase
409 (OADC) (Fisher, Pittsburgh, PA) and 0.05% Tween 80 prior to high-dose mouse aerosol
410 infection. MDR and XDR *M. tuberculosis* clinical isolates representing different
411 resistance phenotypes belong to the collection of strains of the Vall d'Hebron hospital of
412 Barcelona.

413 *M. tuberculosis* H37Rv and H37Rv-Luc were routinely propagated at 37°C in
414 Middlebrook 7H9 broth (Difco) supplemented with 10% Middlebrook albumin-dextrose-
415 catalase (ADC)(Difco), 0.2% glycerol and 0.05% (vol/vol) tyloxapol or on Middlebrook
416 7H10 agar plates (Difco) supplemented with 10% (vol/vol) OADC (Difco). Hygromycin B
417 was added to the medium (50 µg/mL) to ensure plasmid maintenance when propagating
418 the H37Rv-Luc strain. This strain constitutively expresses the luciferase *luc* gene from
419 *Photinus pyralis* (GenBank Accession Number M15077) cloned in a mycobacterial
420 shuttle plasmid derived from pACE-1 (36).

421 **Intracellular MIC assay.** Frozen stocks of macrophage THP-1 cells (ATCC TIB-202)
422 were thawed in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine
423 serum (FBS) (Gibco), 2 mM L-glutamine (Sigma) and 1 mM sodium pyruvate (Sigma).
424 THP-1 cells were passaged only five times and maintained without antibiotics between
425 $2-10 \times 10^5$ cells/mL at 37 °C in a humidified, 5% CO₂ atmosphere. THP-1 cells ($3 \times$
426 10^8) were simultaneously differentiated with phorbol myristate acetate (PMA, 40 ng/mL,
427 Sigma) and infected for 4 hours at a multiplicity of infection (MOI) of 1:1 with a single
428 cell suspension of H37Rv-Luc. After incubation, infected cells were washed four times
429 to remove extracellular bacilli and resuspended (2×10^5 cells/mL) in RPMI medium
430 supplemented with 10% FBS (Hyclone), 2 mM L-glutamine and pyruvate and dispensed
431 in white, flat bottom 384-well plates (Greiner) in a final volume of 50 µL (max. 0.5%
432 DMSO). Plates were incubated for 5 days under 5% CO₂ atmosphere, 37 °C, 80%
433 relative humidity before growth assessment. The Bright-Glo™ Luciferase Assay System
434 (Promega, Madison, WI) was used as cell growth indicator for the H37Rv-Luc strain.
435 Luminescence was measured in an Envision Multilabel Plate Reader (PerkinElmer)

436 using the opaque 384-plate Ultra Sensitive luminescence mode, with a measurement
437 time of 50 ms. A 90% reduction in light production was considered growth inhibition and
438 the IC₉₀ value was interpolated from the dose response curve.

439 **Extracellular MIC assays.** MICs against the H37Rv strain were determined by broth
440 dilution assay in Middlebrook 7H9 medium supplemented with 10% ADC. After
441 incubating at 37 °C for six days, 25 µL resazurin solution (one tablet in 30 mL sterile
442 PBS) was added to each well. Following incubation at 37 °C for two additional days, the
443 lowest concentration of drug that inhibited 90% of resazurin conversion compared to
444 internal DMSO control wells with no drug added was used to define MIC values.

445 MICs against clinical isolates of *M. tuberculosis* were determined using the
446 mycobacteria growth indicator tubes (MGIT) system. Approximately 1 mg wet weight
447 from a Lowenstein-Jensen slant, with an estimated bacterial load of 10⁸ CFU/mL, was
448 inoculated into McCartney vials containing 1 mL of distilled water and 5 glass beads.
449 The mixtures were homogenized by vortexing for 1-3 minutes. The opacity of the
450 suspensions was adjusted by the addition of sterile distilled water to that of a 0.5
451 McFarland turbidity standard. 100 µL were used to inoculate MGIT vials containing
452 serial dilutions of the compounds. MIC values were defined using the BACTEC MGIT
453 960 System (Becton Dickinson) and following the manufacturer's instructions.

454 **HepG2 cytotoxicity assay.** HepG2 cells were cultured using Eagle's minimum
455 essential media (EMEM) supplemented with 10% heat-inactivated FBS, 1% Non-
456 Essential Amino Acid (NEAA), and 1% penicillin/streptomycin. Prior to addition of the
457 cell suspension, 250 nL of test compounds per well were predispensed in Tissue culture
458 -treated black clear-bottomed 384-well plates (Greiner, cat. no. 781091) with an Echo

459 555 instrument. After that, 25 μ L of HepG2 (cat. no. ATCC HB-8065) cells (~3000
460 cells/well) grown to confluency in EMEM supplemented with 10% heat-inactivated FBS,
461 1% NEAA, and 1% penicillin/streptomycin were added to each well with the reagent
462 dispenser. Plates were allowed to incubate at 37 °C with 20% O₂ and 5% CO₂ for 48
463 hrs. After incubation, the plates were equilibrated to room temperature before ATP
464 levels were measured with the CellTiter Glo kit (Promega) as the cell viability read-out.
465 25 μ L of CellTiter Glo substrate dissolved in the buffer was added to each well. Plates
466 were incubated at room temperature for 10 min for stabilization of luminescence signal
467 and read on a View Lux luminometer with excitation and emission filters of 613 and 655
468 nm, respectively. The Tox₅₀ value corresponds to the concentration of the compound
469 necessary to inhibit 50% of cell growth.

470 **Cell Health Assay.** This is a 3-parameter automated imaging cell-based assay to
471 measure the cytotoxic effect of compounds in human liver-derived HepG2 cells. Using
472 fluorescent staining, the key parameters measured in this assay are nuclear size,
473 mitochondrial membrane potential and plasma membrane permeability. HepG2 cells
474 (ATCC HB-8065) were incubated with the test compounds in 384-well plates. After 48
475 hours, the staining cocktail was added. Hoechst 33342 is used to stain nuclei and
476 quantify changes in nuclear morphology. Tetramethylrhodamine, methyl ester (TMRM)
477 is a cationic dye that accumulates in healthy mitochondria that maintain a mitochondrial
478 membrane potential and leaks out of mitochondria when the mitochondrial membrane
479 potential is dissipated. TOTO-3 iodide labels nuclei of permeabilized cells and is used to
480 measure plasma membrane permeability. Following 45 min of incubation with these
481 stains, the plates were sealed using a black seal for reading on an INCell Analyzer 2000

482 (GE Healthcare). Each parameter measurement produces the percentages of cells
483 which are 'LIVE' or 'DEAD'. The IC₅₀ is defined as the compound concentration that
484 inhibits 50% of cell growth.

485 **Ames Assay.** The Ames assay was carried out as previously described (37) using all
486 strains.

487 **hERG Assay.** hERG activity was measured as previously described (38).

488 **Hepatic microsome stability.** Human and animal microsomes and compounds were
489 preincubated at 37 °C prior to addition of NADPH to final protein concentration of 0.5
490 mg/mL and final compound concentration of 0.5 μM. Quantitative analysis was
491 performed using specific LC-MS/MS conditions. The half-life, elimination rate constant,
492 and intrinsic clearance (mL/min/g tissue) were determined. The well-stirred model was
493 used to translate to *in vivo* Cl values (mL/min/Kg).

494 ***In vivo* pharmacokinetics analysis.** Single-dose pharmacokinetics experiments were
495 performed in female C57BL/6 mice, 21-29 g, obtained from Charles River Laboratories
496 (Wilmington, MA) and housed in cages in groups of three animals with water and food
497 *ad libitum*. Animals were maintained for one week before the experiment.

498 The compound was dissolved in 20% Encapsin (Sigma-Aldrich), 5% DMSO (Sigma
499 Aldrich) in saline solution (Sigma Aldrich) for intravenous administration and in 1%
500 methylcellulose (Sigma-Aldrich) in water for oral administration.

501 For PK analysis, 25 μL of tail blood were collected by microsampling at 0.08 h, 0.25 h,
502 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h for intravenous pharmacokinetics and 0.25 h, 0.5
503 h, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h for oral pharmacokinetics.

504 **Assessment of acute efficacy in murine TB models.** Specific pathogen-free, 8-10
505 week-old (18-20 grams) female C57BL/6 mice were purchased from Harlan
506 Laboratories and were allowed to acclimate for one week. The experimental design for
507 the acute assay has been previously described (22). In brief, mice were intratracheally
508 infected with 100,000 CFU/mouse of *M. tuberculosis* H37Rv. Compounds were
509 administered daily for 8 consecutive days starting 24 hours after infection. Lungs were
510 harvested on day 9. All lung lobes were aseptically removed, homogenized and frozen.
511 Homogenates were thawed and plated on 7H11 medium supplemented with 10%
512 OADC plus 0.4% activated charcoal to reduce the effects of compound carryover. CFU
513 were counted after 18 days of incubation at 37 °C. Log₁₀ CFU vs. dose data were
514 plotted. A sigmoidal dose-response curve was fitted and used to estimate ED₉₉ and
515 ED_{max}. Data were analyzed using GraphPad software (Prism). The ED₉₉ was defined as
516 the dose in mg/Kg that reduced the number of CFUs in the lungs of treated mice by
517 99% compared to untreated infected mice. The ED_{max} is the dose in mg/g that resulted
518 in 90% of predicted maximal effect.

519 **Modelling and simulations**

520 The calculated exposures at ED_{max} for **GSK138** and **GSK693** were obtained using the
521 IV mouse PKs profiles fitting to a bicompartamental model to obtain those parameters to
522 simulate the oral whole blood exposures at ED_{max}. Additionally, a monocompartamental
523 model was used to fit the experimental oral pharmacokinetic studies in non-infected
524 mouse together with measured plasma concentrations obtained from the sparse
525 sampling in the Experiment 2 in infected mice. Parameters obtained from this fitting
526 were used to simulate the profile after **GSK693** administration at 100, 200 and 300

527 mg/Kg in the combination study and to calculate the associated exposures (see Table
528 3).

529 **Blood and Plasma pharmacokinetic sampling and analysis**

530 Sample collection from non-infected animals (PK studies): Blood samples (25 μ L) were
531 taken from the lateral tail vein using a micropipette and were mixed, vortexed with 25 μ L
532 of saponin 0.1% and frozen at -80 °C until analysis.

533 Sample collection from infected animals: Mouse tail vein blood was collected at the
534 indicated time points. Briefly, an incision was made in the lateral tail vein. 20-50 μ L of
535 blood was collected in BD Vacutainer® PST™ lithium heparin tubes from each mouse.
536 The tubes were kept on ice before being centrifuged at 8000 rpm for 5 minutes. The
537 supernatant plasma (15-30 μ L) was transferred to labeled microcentrifuge tubes, frozen
538 and stored at -80 °C and then shipped on dry ice to GSK for further analysis.

539 Sample pretreatment and LC-MS/MS analysis: 10 μ L of plasma or blood samples
540 thawed at ambient temperature was mixed with 200 μ L of ACN:MeOH (80:20). After this
541 protein precipitation step, samples were filtered using a 0.45 μ m filter plate (Multiscreen
542 Solvinert 0.45um FTPE, Millipore) and then filtered using a 0.2 μ m filter (AcroPrep
543 Advance 96 Filter Plate 350 μ L, 0.2 μ m PTFE) to ensure sterilization prior to LC-MS
544 analysis.

545 An Acquity Ultra-Performance liquid chromatography (UPLC) system (Waters Corp.,
546 Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (API 4000™, AB
547 Sciex, Foster City, CA, USA) was used for the analysis.

548 The chromatographic separation was conducted at 0.4 mL/min in an Acquity UPLC™
549 BEH C18 column (50x2.1 mm i.d., 1.7 mm; Waters Corp.) at 40°C with acetonitrile
550 (ACN) (SigmaAldrich) and 0.1% formic acid as eluents.

551 Sciex Analyst software was used for the data analysis. The non-compartmental data
552 analysis (NCA) was performed with Phoenix WinNonlin software in order to determine
553 pharmacokinetic parameters and exposure.

554 **High-dose aerosol mouse infection model.** Female specific pathogen-free BALB/c
555 mice, aged 5-6 weeks, were purchased from Charles River (Wilmington, MA). Mice
556 were infected by aerosol using the Inhalation Exposure System (Glas-col, Terre Haute,
557 IN) using a log phase culture of *M. tuberculosis* H37Rv with an OD₆₀₀ of 0.8-1 to implant
558 approximately 3.5-4 log₁₀ CFU in the lungs. Treatment started 2 weeks later (D0). Mice
559 were sacrificed for lung CFU counts the day after infection and on D0 to determine the
560 number of CFU implanted and the number present at the start of treatment,
561 respectively.

562 **Antibiotic treatment.** Mice were treated with the drugs and drug combinations
563 indicated in Figures 2 through 6 at the following doses (in mg/Kg body weight):
564 bedaquiline (25), pretomanid (50 or 100), moxifloxacin (100), linezolid (100), isoniazid
565 (10), rifampicin (10), sutezolid (50), **GSK138** (50, 100, or 200), **GSK693** (100, 200, or
566 300), GSK656 (10), GSK286 (50), NITD-113 (150), and pyrazinamide (150). **GSK693**,
567 **GSK138**, and GSK286 were formulated in 1% methylcellulose solution. GSK656 was
568 formulated in distilled water. Other drugs were formulated as previously described (39-
569 41). Bedaquiline and pretomanid were administered in back-to-back gavages and

570 separated from companion drugs by at least 3 hours. Rifampicin was administered
571 alone at least one hour before any companion drug.

572 **Evaluation of drug efficacy.** Efficacy determinations were based on lung CFU counts
573 after 4 or 8 weeks of treatment and, in one experiment, cohorts of mice were also kept
574 for 12 weeks after completing 8 or 12 weeks of treatment to assess for relapse-free
575 cure. At each time point, lungs were removed aseptically and homogenized in 2.5 mL of
576 PBS. Serial 10-fold dilutions of lung homogenate were plated on selective 7H11 agar
577 plates. To assess for relapse-free cure, the entire lung homogenate was plated. In
578 experiments with bedaquiline, lung homogenates were plated on 7H11 agar
579 supplemented with 0.4% activated charcoal to reduce drug carryover and doubling the
580 concentrations of selective antibiotics in the media to mitigate binding to charcoal.

581 **Statistical analysis.** Group means were compared by one-way ANOVA with Dunnett's
582 correction for multiple comparisons or by Student's t-test, as appropriate, using
583 GraphPad Prism version 8.

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593 REFERENCES

- 594 1. World Health Organization. 2023. Global Tuberculosis Report 2023. Geneva
595 2. **Pai M, Kasaeva T, Swaminathan S.** 2022. Covid-19's Devastating Effect on
596 Tuberculosis Care - A Path to Recovery. *N Engl J Med* **386**:1490-1493.
597 3. **Bernstein J, Lott WA, Steinberg BA, Yale HL.** 1952. Chemotherapy of experimental
598 tuberculosis. V. Isonicotinic acid hydrazide (nydrazid) and related compounds. *Am Rev*
599 *Tuberc* **65**:357-364.
600 4. **Zhang Y, Heym B, Allen B, Young D, Cole S.** 1992. The catalase-peroxidase gene and
601 isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* **358**:591-593.
602 5. **Quémard A, Sacchetti JC, Dessen A, Vilcheze C, Bittman R, Jacobs WR, Jr.,**
603 **Blanchard JS.** 1995. Enzymatic characterization of the target for isoniazid in
604 *Mycobacterium tuberculosis*. *Biochemistry* **34**:8235-8241.
605 6. **Rozwarski DA, Grant GA, Barton DH, Jacobs WR, Jr., Sacchetti JC.** 1998.
606 Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium*
607 *tuberculosis*. *Science* **279**:98-102.
608 7. **Vilchèze C, Wang F, Arai M, Hazbón MH, Colangeli R, Kremer L, Weisbrod TR,**
609 **Alland D, Sacchetti JC, Jacobs WR, Jr.** 2006. Transfer of a point mutation in
610 *Mycobacterium tuberculosis inhA* resolves the target of isoniazid. *Nat Med* **12**:1027-
611 1029.
612 8. **Dean AS, Zignol M, Cabibbe AM, Falzon D, Glaziou P, Cirillo DM, Köser CU,**
613 **Gonzalez-Angulo LY, Tosas-Auget O, Ismail N, Tahseen S, Ama MCG, Skrahina A,**
614 **Alikhanova N, Kamal SMM, Floyd K.** 2020. Prevalence and genetic profiles of
615 isoniazid resistance in tuberculosis patients: A multicountry analysis of cross-sectional
616 data. *PLoS Med* **17**:e1003008.
617 9. **Fregonese F, Ahuja SD, Akkerman OW, Arakaki-Sanchez D, Ayakaka I, Baghaei P,**
618 **Bang D, Bastos M, Benedetti A, Bonnet M, Cattamanchi A, Cegielski P, Chien JY,**
619 **Cox H, Dedicoat M, Erkens C, Escalante P, Falzon D, Garcia-Prats AJ, Gegia M,**
620 **Gillespie SH, Glynn JR, Goldberg S, Griffith D, Jacobson KR, Johnston JC, Jones-**
621 **López EC, Khan A, Koh WJ, Kritski A, Lan ZY, Lee JH, Li PZ, Maciel EL, Galliez**
622 **RM, Merle CSC, Munang M, Narendran G, Nguyen VN, Nunn A, Ohkado A, Park**
623 **JS, Phillips PPJ, Ponnuraja C, Reves R, Romanowski K, Seung K, Schaaf HS,**
624 **Skrahina A, Soolingen DV, et al.** 2018. Comparison of different treatments for
625 isoniazid-resistant tuberculosis: an individual patient data meta-analysis. *Lancet Respir*
626 *Med* **6**:265-275.
627 10. **Gegia M, Winters N, Benedetti A, van Soolingen D, Menzies D.** 2017. Treatment of
628 isoniazid-resistant tuberculosis with first-line drugs: a systematic review and meta-
629 analysis. *Lancet Infect Dis* **17**:223-234.
630 11. **Stagg HR, Harris RJ, Hatherell HA, Obach D, Zhao H, Tsuchiya N, Kranzer K,**
631 **Nikolayevskyy V, Kim J, Lipman MC, Abubakar I.** 2016. What are the most
632 efficacious treatment regimens for isoniazid-resistant tuberculosis? A systematic review
633 and network meta-analysis. *Thorax* **71**:940-949.
634 12. **Stagg HR, Lipman MC, McHugh TD, Jenkins HE.** 2017. Isoniazid-resistant
635 tuberculosis: a cause for concern? *Int J Tuberc Lung Dis* **21**:129-139.
636 13. **Sulis G, Pai M.** 2020. Isoniazid-resistant tuberculosis: A problem we can no longer
637 ignore. *PLoS Med* **17**:e1003023.

- 638 14. **World Health Organization.** 2018. WHO treatment guidelines for isoniazid- resistant
639 tuberculosis. Supplement to the WHO treatment guidelines for drug-resistant
640 tuberculosis. Geneva.
- 641 15. **Brossier F, Boudinet M, Jarlier V, Petrella S, Sougakoff W.** 2016. Comparative study
642 of enzymatic activities of new KatG mutants from low- and high-level isoniazid-resistant
643 clinical isolates of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* **100**:15-24.
- 644 16. **Dooley KE, Miyahara S, von Groote-Bidlingmaier F, Sun X, Hafner R, Rosenkranz
645 SL, Ignatius EH, Nuermberger EL, Moran L, Donahue K, Swindells S, Vanker N,
646 Diacon AH.** 2020. Early Bactericidal Activity of Different Isoniazid Doses for Drug
647 Resistant TB (INHindsight): A Randomized Open-label Clinical Trial. *Am J Respir Crit
648 Care Med* **201**:1416-1424.
- 649 17. **Seifert M, Catanzaro D, Catanzaro A, Rodwell TC.** 2015. Genetic mutations
650 associated with isoniazid resistance in *Mycobacterium tuberculosis*: a systematic review.
651 *PLoS One* **10**:e0119628.
- 652 18. **Rožman K, Sosič I, Fernandez R, Young RJ, Mendoza A, Gobec S, Encinas L.** 2017.
653 A new 'golden age' for the antitubercular target InhA. *Drug Discov Today* **22**:492-502.
- 654 19. **Manjunatha UH, SP SR, Kondreddi RR, Noble CG, Camacho LR, Tan BH, Ng SH,
655 Ng PS, Ma NL, Lakshminarayana SB, Herve M, Barnes SW, Yu W, Kuhen K,
656 Blasco F, Beer D, Walker JR, Tonge PJ, Glynne R, Smith PW, Diagana TT.** 2015.
657 Direct inhibitors of InhA are active against *Mycobacterium tuberculosis*. *Sci Transl Med*
658 **7**:269ra263.
- 659 20. **Martínez-Hoyos M, Perez-Herran E, Gulten G, Encinas L, Álvarez-Gómez D,
660 Alvarez E, Ferrer-Bazaga S, García-Pérez A, Ortega F, Angulo-Barturen I, Rullas-
661 Trincado J, Blanco Ruano D, Torres P, Castañeda P, Huss S, Fernández Menéndez
662 R, González Del Valle S, Ballell L, Barros D, Modha S, Dhar N, Signorino-Gelo F,
663 McKinney JD, García-Bustos JF, Lavandera JL, Sacchetti J, Jimenez MS,
664 Martín-Casabona N, Castro-Pichel J, Mendoza-Losana A.** 2016. Antitubercular drugs
665 for an old target: GSK693 as a promising InhA direct inhibitor. *EBioMedicine* **8**:291-
666 301.
- 667 21. **Xia Y, Zhou Y, Carter DS, McNeil MB, Choi W, Halladay J, Berry PW, Mao W,
668 Hernandez V, O'Malley T, Korkegian A, Sunde B, Flint L, Woolhiser LK,
669 Scherman MS, Gruppo V, Hastings C, Robertson GT, Ioerger TR, Sacchetti J,
670 Tonge PJ, Lenaerts AJ, Parish T, Alley M.** 2018. Discovery of a cofactor-independent
671 inhibitor of *Mycobacterium tuberculosis* InhA. *Life Sci Alliance* **1**:e201800025.
- 672 22. **Rullas J, García JJ, Beltrán M, Cardona PJ, Cáceres N, García-Bustos JF, Angulo-
673 Barturen I.** 2010. Fast standardized therapeutic-efficacy assay for drug discovery against
674 tuberculosis. *Antimicrob Agents Chemother* **54**:2262-2264.
- 675 23. **Nuermberger EL.** 2017. Preclinical Efficacy Testing of New Drug Candidates.
676 *Microbiol Spectr* **5**.
- 677 24. **Almeida D, Ioerger T, Tyagi S, Li SY, Mdluli K, Andries K, Grosset J, Sacchetti J,
678 Nuermberger E.** 2016. Mutations in pepQ Confer Low-Level Resistance to Bedaquiline
679 and Clofazimine in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*
680 **60**:4590-4599.
- 681 25. **Grosset J, Truffot-Pernot C, Lacroix C, Ji B.** 1992. Antagonism between isoniazid and
682 the combination pyrazinamide-rifampin against tuberculosis infection in mice.
683 *Antimicrob Agents Chemother* **36**:548-551.

- 684 26. **Rosenthal IM, Williams K, Tyagi S, Peloquin CA, Vernon AA, Bishai WR, Grosset**
685 **JH, Nuermberger EL.** 2006. Potent twice-weekly rifapentine-containing regimens in
686 murine tuberculosis. *Am J Respir Crit Care Med* **174**:94-101.
- 687 27. **Li X, Hernandez V, Rock FL, Choi W, Mak YSL, Mohan M, Mao W, Zhou Y,**
688 **Easom EE, Plattner JJ, Zou W, Pérez-Herrán E, Giordano I, Mendoza-Losana A,**
689 **Alemparte C, Rullas J, Angulo-Barturen I, Crouch S, Ortega F, Barros D, Alley**
690 **MRK.** 2017. Discovery of a Potent and Specific *M. tuberculosis* Leucyl-tRNA
691 Synthetase Inhibitor: (S)-3-(Aminomethyl)-4-chloro-7-(2-
692 hydroxyethoxy)benzo[c][1,2]oxaborol-1(3H)-ol (GSK656). *J Med Chem* **60**:8011-8026.
- 693 28. **Tenero D, Derimanov G, Carlton A, Tonkyn J, Davies M, Cozens S, Gresham S,**
694 **Gaudion A, Puri A, Muliaditan M, Rullas-Trincado J, Mendoza-Losana A,**
695 **Skingsley A, Barros-Aguirre D.** 2019. First-Time-in-Human Study and Prediction of
696 Early Bactericidal Activity for GSK3036656, a Potent Leucyl-tRNA Synthetase Inhibitor
697 for Tuberculosis Treatment. *Antimicrob Agents Chemother* **63**.
- 698 29. **Nuermberger EL, Martínez-Martínez MS, Sanz O, Urones B, Esquivias J, Soni H,**
699 **Tasneen R, Tyagi S, Li SY, Converse PJ, Boshoff HI, Robertson GT, Besra GS,**
700 **Abrahams KA, Upton AM, Mdluli K, Boyle GW, Turner S, Fotouhi N, Cammack**
701 **NC, Siles JM, Alonso M, Escribano J, Lelievre J, Rullas-Trincado J, Pérez-Herrán**
702 **E, Bates RH, Maher-Edwards G, Barros D, Ballell L, Jiménez E.** 2022. GSK2556286
703 Is a Novel Antitubercular Drug Candidate Effective In Vivo with the Potential To
704 Shorten Tuberculosis Treatment. *Antimicrob Agents Chemother* **66**:e0013222.
- 705 30. **Wakamoto Y, Dhar N, Chait R, Schneider K, Signorino-Gelo F, Leibler S,**
706 **McKinney JD.** 2013. Dynamic persistence of antibiotic-stressed mycobacteria. *Science*
707 **339**:91-95.
- 708 31. **Conradie F, Bagdasaryan TR, Borisov S, Howell P, Mikiashvili L, Ngubane N,**
709 **Samoilova A, Skornykova S, Tudor E, Variava E, Yablonskiy P, Everitt D, Wills**
710 **GH, Sun E, Olugbosi M, Egizi E, Li M, Holsta A, Timm J, Bateson A, Crook AM,**
711 **Fabiane SM, Hunt R, McHugh TD, Tweed CD, Foraida S, Mendel CM, Spigelman**
712 **M.** 2022. Bedaquiline-Pretomanid-Linezolid Regimens for Drug-Resistant Tuberculosis.
713 *N Engl J Med* **387**:810-823.
- 714 32. **Conradie F, Diacon AH, Ngubane N, Howell P, Everitt D, Crook AM, Mendel CM,**
715 **Egizi E, Moreira J, Timm J, McHugh TD, Wills GH, Bateson A, Hunt R, Van**
716 **Niekerk C, Li M, Olugbosi M, Spigelman M.** 2020. Treatment of Highly Drug-
717 Resistant Pulmonary Tuberculosis. *N Engl J Med* **382**:893-902.
- 718 33 **Nyang'wa BT.** 2021. SP-34 TB-PRACTECAL Stage 2 Trial Efficacy Results, abstr 52nd
719 World Conference on Lung Health of the International Union Against Tuberculosis and
720 Lung Disease (The Union), Paris, 22 October, 2021.
- 721 34. **Polli JW, Wring SA, Humphreys JE, Huang L, Morgan JB, Webster LO, Serabjit-**
722 **Singh CS.** 2001. Rational use of in vitro P-glycoprotein assays in drug discovery. *J*
723 *Pharmacol Exp Ther* **299**:620-628.
- 724 35. **Hill AP, Young RJ.** 2010. Getting physical in drug discovery: a contemporary
725 perspective on solubility and hydrophobicity. *Drug Discov Today* **15**:648-655.
- 726 36. **Sorrentino F, Gonzalez del Rio R, Zheng X, Presa Matilla J, Torres Gomez P,**
727 **Martinez Hoyos M, Perez Herran ME, Mendoza Losana A, Av-Gay Y.** 2016.
728 Development of an Intracellular Screen for New Compounds Able To Inhibit

- 729 *Mycobacterium tuberculosis* Growth in Human Macrophages. Antimicrob Agents
730 Chemother **60**:640-645.
- 731 37. **Maron DM, Ames BN.** 1983. Revised methods for the Salmonella mutagenicity test.
732 Mutat Res **113**:173-215.
- 733 38. **Gillie DJ, Novick SJ, Donovan BT, Payne LA, Townsend C.** 2013. Development of a
734 high-throughput electrophysiological assay for the human ether-à-go-go related
735 potassium channel hERG. J Pharmacol Toxicol Methods **67**:33-44.
- 736 39. **Lounis N, Veziris N, Chauffour A, Truffot-Pernot C, Andries K, Jarlier V.** 2006.
737 Combinations of R207910 with drugs used to treat multidrug-resistant tuberculosis have
738 the potential to shorten treatment duration. Antimicrob Agents Chemother **50**:3543-3547.
- 739 40. **Tyagi S, Nuermberger E, Yoshimatsu T, Williams K, Rosenthal I, Lounis N, Bishai**
740 **W, Grosset J.** 2005. Bactericidal activity of the nitroimidazopyran PA-824 in a murine
741 model of tuberculosis. Antimicrob Agents Chemother **49**:2289-2293.
- 742 41. **Williams KN, Stover CK, Zhu T, Tasneen R, Tyagi S, Grosset JH, Nuermberger E.**
743 2009. Promising antituberculosis activity of the oxazolidinone PNU-100480 relative to
744 that of linezolid in a murine model. Antimicrob Agents Chemother **53**:1314-1319.

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