



## 21 **Synopsis**

22 *Mycobacterium tuberculosis* is a deadly human pathogen that latently infects a third of the world's  
23 population, resulting in approximately 1.5 million deaths per year. Due to the difficulties and  
24 expense of carrying out animal drug trials using *M. tuberculosis* and rodents, infections of the  
25 zebrafish *Danio rerio* with *M. marinum* have been used as a surrogate. However the methods so far  
26 described require specialised equipment and a high level of operator expertise.

27 We investigated a natural infection model where zebrafish embryos are infected through incubation  
28 in media containing *M. marinum*. Using bioluminescently labelled *M. marinum*, we have  
29 characterised the nature of infection and established a model for interventional drug therapy. We  
30 have used a selection of traditional and experimental compounds to validate this model for anti-  
31 mycobacterial drug discovery. We observed that only three of the six treatments tested (Delamanid,  
32 SN30527 and rifampicin) retarded the growth of *M. marinum* in vitro. In contrast, five of the six  
33 treatments (Pretomanid, Delamanid, SN30488, SN30527 and rifampicin) retarded the growth of *M.*  
34 *tuberculosis* in vitro. Importantly, these same five treatments significantly reduced the  
35 bioluminescent signal from naturally infected zebrafish embryos.

36 Overall this study has demonstrated that zebrafish embryos naturally infected with bioluminescent  
37 *M. marinum* can be used for the rapid screening of anti-mycobacterial compounds with readily  
38 available equipment and limited expertise. The result is an assay that can be carried out by a wide  
39 variety of laboratories for minimal cost and without high levels of zebrafish expertise.

40

## 41 Introduction

42 *Mycobacterium tuberculosis* is a deadly human pathogen that latently infects a third of the world's  
43 population. Around 5-10% of these infections develop into active disease, resulting in approximately  
44 1.5 million deaths per year<sup>1</sup>. Tuberculosis (TB) is treatable with antibiotics, albeit with extended  
45 treatment times required and a high financial cost<sup>1</sup>, and as a result has become less prevalent in the  
46 developed world, although it remains a major health issue globally<sup>1</sup>. However the emergence of  
47 multi drug resistant (MDR-TB) and totally drug resistant (TDR-TB) isolates of *M. tuberculosis* has once  
48 again brought TB into the spotlight for all countries and has necessitated the development of novel  
49 drugs to combat this pathogen<sup>2, 3</sup>. A change in the global disease burden of HIV/AIDS has also added  
50 impetus to this cause; the number of people co-infected with HIV and *M. tuberculosis* is rapidly  
51 increasing globally, resulting in a destructive synergy which exponentially exacerbates the disease  
52 progression of both diseases<sup>4</sup>.

53 Due to the difficulties and dangers involved in culturing *M. tuberculosis*, an airborne Biosafety Level  
54 3 pathogen, faster-growing and less pathogenic mycobacterial species, such as *M. smegmatis* and *M.*  
55 *marinum*, are routinely exploited for TB research and anti-mycobacterial drug discovery<sup>5-8</sup>. *M.*  
56 *marinum* is a pathogen of ectotherms (fish, amphibians and reptiles) that produces a tuberculosis-  
57 like disease<sup>9</sup>. *M. marinum* is a close genetic relative of *M. tuberculosis*, with which it shares  
58 conserved virulence determinants<sup>9</sup>, and has been known to cause granulomatous skin infections in  
59 humans<sup>10, 11</sup>. Infection of the tropical zebrafish, *Danio rerio*, with *M. marinum* has been used to  
60 develop a surrogate in vivo model of TB pathogenesis<sup>12</sup> useful for the rapid screening of potential  
61 antimycobacterial compounds<sup>13</sup>. Zebrafish are genetically tractable<sup>9</sup> and possess a complex immune  
62 system comparable to that of humans<sup>14-16</sup>. As a result, zebrafish have been extensively used for  
63 disease modelling and drug discovery for both communicable and non-communicable diseases<sup>13, 17-</sup>  
64 <sup>24</sup>. Infection of zebrafish with *M. marinum* through microinjection results in the development of  
65 necrotic granulomatous lesions reminiscent of human TB infection,<sup>25, 26</sup> lending weight to the use of  
66 this model host as a surrogate for mycobacterial research and drug discovery. One drawback of the

67 previously published infection protocols is that they require specialised equipment and a high level  
68 of operator expertise<sup>13</sup>.

69 Bioluminescence is a biological reaction which results in the production of light via a luciferase  
70 catalysed reaction. It is a naturally occurring process with several variants seen across several  
71 kingdoms and has been harnessed as a reporter in both in vitro and in vivo assays<sup>27, 28</sup>  
72 Bioluminescence allows for non-invasive monitoring of luciferase-expressing bacteria within a host,  
73 as the light produced by the bacterium travels through the host tissues and can be readily detected  
74 <sup>28-30</sup>. As tagged cells only produce a signal when alive, bioluminescence is an excellent reporter to  
75 rapidly assay for antimicrobial compounds, non-destructively and in real-time, in microtitre plate  
76 formats using a luminometer, or in vivo using sensitive imaging equipment<sup>28, 31-33</sup>.

77 Zebrafish and other fish readily become naturally infected with *M. marinum* present in their  
78 environment<sup>34</sup>. However it is not possible to tell which fish are infected at an early stage without  
79 euthanising the animal and plating out for viable bacteria<sup>26</sup>. Here we use bioluminescently tagged *M.*  
80 *marinum* M to establish natural infections in zebrafish embryos, utilising the light emitted by these  
81 bacteria to identify which fish have become infected. We show that naturally infected fish can be  
82 treated with potential anti-mycobacterial compounds and light output used as an indicator of in vivo  
83 drug efficacy.

84

## 85 **Materials and Methods**

### 86 *Bacterial strains and plasmids*

87 Bacterial strains and plasmids used throughout this study are described in Table 1. Bacteria were  
88 transformed as previously described<sup>35</sup>. *M. marinum* strains were grown at 28°C and *M. tuberculosis*  
89 at 37°C, with shaking at 200rpm and 100rpm respectively, in Middlebrook 7H9 broth (Fort Richard)  
90 supplemented with 10% ADC enrichment media (Fort Richard) and 0.5% glycerol (Sigma Aldrich)  
91 under the appropriate antibiotic selection (kanamycin at 25µg ml<sup>-1</sup> and hygromycin at 50µg ml<sup>-1</sup>  
92 [Sigma Aldrich]).

93

### 94 *In vitro drug testing*

95 *M. marinum* BSG101 and *M. tuberculosis* BSG001 were grown without antibiotic selection to mid log  
96 phase, then diluted to an optical density at 600nm (OD<sub>600</sub>) of 0.01. Bacteria were aliquoted (100µl,  
97 5x10<sup>5</sup>cfu ml<sup>-1</sup> approx.) into the wells of a black 96 well microtitre plate (Grenier Bio-One). Test  
98 compounds were dissolved in DMSO, added 1:100 to the top most well and then diluted two-fold in  
99 a series down the plate (Table 2). The plates were incubated at 28°C (*M. marinum*) or 37°C (*M.*  
100 *tuberculosis*) and bioluminescence monitored daily over 7 days using a Victor X1 luminometer  
101 (Perkin Elmer).

102

### 103 *Fish husbandry*

104 Zebrafish (*Danio rerio*) embryos were obtained from natural spawnings and raised at 28°C in E3  
105 Medium (0.33 mM calcium chloride, 0.33 mM magnesium sulphate, 0.14mM potassium chloride and  
106 5 mM sodium chloride). The medium was supplemented with 0.003% phenylthiourea (PTU) to inhibit  
107 pigmentation when embryos were being imaged. Zebrafish embryos of the age used do not fall

108 under the New Zealand Animal Welfare Act 1999 and so experiments did not require approval from  
109 an animal ethics committee.

110

#### 111 *Natural infection of zebrafish embryos*

112 *M. marinum* strains were grown to mid log phase (OD<sub>600</sub> between 0.8 and 2) without antibiotic  
113 selection, washed once in E3 before being adjusted to an OD<sub>600</sub> of 1. The approximate concentration  
114 should be around  $5 \times 10^8$  cfu ml<sup>-1</sup>; this was confirmed by retrospectively plating inocula onto 7H11  
115 supplemented with 10% OADC and 0.5% glycerol. Zebrafish embryos at 2 days post-fertilisation (dpf)  
116 were dechorionated, either manually or using pronase, as previously described<sup>36</sup>. Groups of embryos  
117 (50-300) were then placed in 9mm petri dishes containing 25ml of E3 supplemented with varying  
118 concentrations of *M. marinum* (by varying the volume of adjusted bacteria) and incubated for 4 days  
119 at 28°C. After infection, any non-internalised bacteria were removed by gently washing embryos  
120 four times with fresh E3 in groups of 10 in separate wells of a 24 well tissue culture plates (BD  
121 Falcon) using gentle aspiration of the media. Embryos were left in the 24 well plate overnight to  
122 remove transient bacteria and then rinsed 4 times in E3 to remove the transient population.  
123 Embryos were then individually placed into the wells of a clear-bottomed black 96 well microtitre  
124 plate (Nunc) with 100µl of E3 and prepared for drug treatment.

125

#### 126 *Injection of zebrafish embryos*

127 *M. marinum* strains were prepared as above. Zebrafish embryos 2dpf were manually de-  
128 chorionated, anaesthetised using 0.168 mg ml<sup>-1</sup> tricaine (Sigma-Aldrich) in E3 medium<sup>37</sup> and infected  
129 by microinjection into the caudal vein as previously described<sup>38</sup>.

130

#### 131 *Measurement of bioluminescence from zebrafish embryos*

132 All bioluminescent measurements were carried out using a Victor X1 luminometer (Perkin Elmer)  
133 with a 1 second exposure time on an open filter. Bioluminescence from zebrafish infected with *M.*  
134 *marinum* BSG100 was visualised after addition of luciferin ( $30\mu\text{g ml}^{-1}$ ) (Gold Biotechnology).

135

#### 136 *Drug testing in zebrafish embryos*

137 Infected zebrafish embryo were read on a luminometer after removal of transient bacteria and  
138 detectably infected embryos (RLU >20) were randomly distributed within drug treatment groups.  
139 Drugs were made up to 100x working concentration in an appropriate solvent. They were diluted  
140 1:10 in E3 and  $10\mu\text{l}$  of diluted drug was placed into each well of the 96 well plate containing  $100\mu\text{l}$  of  
141 E3. The final concentration of the test compounds was  $10\mu\text{M}$ . E3 was used as a no treatment  
142 control. Embryos were treated at 5 days post infection and incubated at  $28^{\circ}\text{C}$  during treatment.

143

#### 144 *Microscopy*

145 For imaging on the fluorescent inverted microscope, embryos were anaesthetised in tricaine as  
146 above and mounted in 3% (w/v) methylcellulose in E3 to reduce embryo movement. Images were  
147 captured on a Nikon SMZ1500 microscope with NIS-Elements F version 4.00.06 software.

148

#### 149 *Statistics*

150 Data analysis was performed as indicated in the figure legends using the GraphPadPrism (version 5)  
151 package.

152 **Results**

153 **Luminescence can be used to monitor zebrafish embryos naturally infected with luciferase-tagged**  
154 ***M. marinum***

155 To determine if zebrafish embryos infected with bioluminescently labelled *M. marinum* could be  
156 monitored using a luminometer, we incubated embryos (n=300) aged 1 and 2 dpf in E3 containing  
157  $1 \times 10^7$  cfu ml<sup>-1</sup> of *M. marinum* tagged either with a modified firefly luciferase (designated *M.*  
158 *marinum* BSG100) or with a modified bacterial luciferase (designated *M. marinum* BSG101). At  
159 different time points, we removed the embryos from the *M. marinum*-containing media, washed  
160 them in fresh media and measured light. As the firefly luciferase reaction requires luciferin as a  
161 substrate, we added this exogenously (30 µg ml<sup>-1</sup>) when embryos were placed into the 96 well  
162 plates. Bioluminescence from infected embryos did not rise above background levels until 4 days  
163 post infection, with comparable levels of light emitted from BSG100 and BSG101 infected embryos  
164 (Fig. 1). Natural infection of embryos with *M. marinum* did not result in any premature deaths when  
165 compared to the uninfected group. While embryos infected with *M. marinum* labelled with the  
166 firefly luciferase (BSG100) produced more light (median maximum value of 709 relative light units  
167 [RLU] (ranging from 328 to 2872)) compared to the bacterial luciferase tagged strain (BSG101)  
168 (median maximum value of 446 RLU (ranging from 122 to 796)), the exogenous addition of luciferin  
169 increased the time required to carry out the assay and the expense of the technique. We therefore  
170 selected bacterial-luciferase tagged *M. marinum* M (BSG101) for further study.

171

172 **Natural infection results in gill colonisation and transient gut colonisation**

173 We investigated the nature and location of natural *M. marinum* infection of embryos using a strain  
174 of *M. marinum* expressing the red fluorescent protein tdTomato<sup>13</sup> (designated BSG102). We  
175 incubated the embryos with BSG102 for 4 days and then determined the location of the infecting  
176 bacteria using a fluorescent microscope (Fig. 2). We observed fluorescently tagged bacteria

177 throughout the digestive tract, clustering around the developing gills and lower jaw of the larvae  
178 (Fig. 2 A&B). We speculated that those bacteria present within the digestive tract could represent a  
179 transient colonisation. These bacteria were seen to be removed following defecation. To test this,  
180 we washed infected embryos in fresh media and incubated for a further 24 hours. After this  
181 incubation period, we observed that the bacteria that were previously present within the digestive  
182 tract had gone, while the bacteria associated with the developing gills were still present (Fig. 2C). We  
183 observed that the digestive tract remained clear of detectable bacteria for the majority of the  
184 experiment, with some non-transient colonisation appearing approximately 11 days post infection.

185

#### 186 **Optimisation of infectious dose and infection protocol**

187 In order to determine the minimum dose for establishing a traceable infection within a single  
188 zebrafish embryo, we incubated embryos with concentrations of *M. marinum* BSG101 ranging from  
189  $1 \times 10^4$  to  $1 \times 10^7$  cfu ml<sup>-1</sup> and followed bioluminescence over 12 days using a luminometer. A dose of  
190  $1 \times 10^7$  cfu ml<sup>-1</sup> BSG101 was the only one tested that established an infection that we could reliably  
191 detect above background levels using our luminometer (Fig. 3). This dose resulted in approximately  
192 40-60% of the exposed embryos becoming bioluminescent, depending on the experiment (data not  
193 shown). When embryos were exposed to a dual bioluminescent/fluorescent tagged strain of *M.*  
194 *marinum* (BSG103) and examined by fluorescence microscopy, we observed that more than 90% of  
195 the embryos were infected, but only half of these could be detected by luminometry (data not  
196 shown).

197 In an effort to reduce the time required to process embryos for infection, we investigated the effect  
198 of manual versus chemical dechoriation (treatment with pronase). We observed that pronase  
199 treatment reduced the proportion of infected embryos detectable by luminometry (45% for manual,  
200 7% for pronase) and light levels from infected embryos were also consistently lower (20-150 RLU for  
201 manual, 20-76 RLU for pronase). For these reasons, we adopted the following optimised protocol for

202 further experiments: 1) manual dechoriation of embryos 2 dpf, 2) immersion in media containing  
203  $1 \times 10^7$  cfu ml<sup>-1</sup> *M. marinum* for 4 days at 28°C, 3) wash in fresh media and incubation for a further 24  
204 hours at 28°C, 4) transfer of individual embryos to the wells of a black 96 well plate using a sterile  
205 plastic pasteur pipette for measurement of light and drug intervention studies.

206

## 207 **Drug treatment of naturally infected zebrafish embryos**

208 Zebrafish embryos, infected using the optimised natural infection protocol, were treated with  
209 rifampicin and a variety of nitroimidazole-based next generation and experimental anti-  
210 mycobacterial drugs (Table 2, Fig. 4). Delamanid was recently approved for clinical use, while  
211 Pretomanid is in human Phase III combination trials<sup>39</sup>. The experimental compounds SN30488,  
212 SN30527 and SN30982<sup>40</sup> are analogues of Pretomanid with varying lipophilic side chains, selected for  
213 their wide range of potencies against *M. tuberculosis* cultures. For comparison, we treated embryos  
214 infected through microinjection into the caudal vein with a subset of the compounds (Fig. 4). We  
215 considered all injected embryos as infected, whereas we only selected embryos with observable  
216 light emission after natural infection for use in drug intervention studies. For naturally infected  
217 embryos we used reduction in light emission as a surrogate measure of anti-mycobacterial activity;  
218 for injected embryos, we measured drug efficacy by embryo survival. We also exposed in vitro  
219 grown *M. marinum* and *M. tuberculosis* to the same compounds.

220 We observed that at the concentration used, only three of the six treatments tested (Delamanid,  
221 SN30527 and rifampicin) retarded the growth of *M. marinum* BSG101 in vitro (as shown by a lack of  
222 increase in bioluminescence) (Fig 5A). In contrast, five of the six treatments tested (Pretomanid,  
223 Delamanid, SN30488, SN30527 and rifampicin) retarded the growth of *M. tuberculosis* BSG001 in  
224 vitro (Fig 5B). Interestingly the same five treatments tested (Pretomanid, Delamanid, SN30488,  
225 SN30527 and rifampicin) significantly reduced the bioluminescent signal from naturally infected  
226 zebrafish embryos ( $p < 0.001$ , Kruskal Wallis test with Dunns multiple comparisons) (Fig. 4A). The data

227 is summarised in Table 3. Similarly, we observed a 90% survival rate for BSG101 injected embryos  
228 treated with Pretomanid and Delamanid, compared to 20% survival of untreated injected embryos  
229 or those treated with SN30982 or rifampicin (Fig. 4B).

230

## 231 Discussion

232 There is a clear and desperate need for new medicines to treat TB. As working with *M. tuberculosis*  
233 limits research and preclinical drug development to those laboratories around the world with the  
234 resources and facilities to safely handle the bacterium, non-tuberculous mycobacteria such as *M.*  
235 *marinum* are widely used as surrogates<sup>5, 7, 41, 42</sup>. A major drawback of using *M. marinum* for  
236 antimycobacterial compound screening is that the in vitro resistance profile of the bacterium is very  
237 different to *M. tuberculosis*. This difference can be observed in the drastically different MIC values  
238 for the compounds tested against the two organisms in this study (Table 2). Two of the compounds  
239 (Pretomanid and SN30488) were not effective against *M. marinum* but were effective against *M.*  
240 *tuberculosis*. This means that screening compounds for activity against *M. marinum* in vitro runs the  
241 risk of missing potential anti-TB agents.

242 Infection of zebrafish, either as adults or as embryos, by injection with *M. marinum* has proved to be  
243 a useful model for studying mycobacterial pathogenicity and for drug screening<sup>13, 43, 44</sup>. However,  
244 current infection protocols require specialised equipment and a high level of operator expertise. We  
245 first wanted to establish whether it was possible to infect zebrafish embryos by exposure to *M.*  
246 *marinum* in the media, and to monitor infection dynamics using bioluminescence. We determined  
247 that incubation for four days in a petri-dish containing  $10^7$  cfu of *M. marinum* per ml of media was  
248 sufficient to establish an infection. Indeed, microscopic imaging demonstrated that fluorescent *M.*  
249 *marinum* could be visualised in the developing gills and digestive tract after this time.

250 The kinetics of natural infection we observed fits with existing understanding of zebrafish larval  
251 development; their mouth has been demonstrated to open from 3 dpf and their gut exists as an

252 open-ended tube by 4 dpf<sup>45</sup>. The initial digestive tract colonisation was found to be transitory,  
253 although at later stages a non-transitory colonisation was observed. It is unsurprising that the  
254 embryos could be infected via bathing, as this should represent one of the ways that zebrafish could  
255 become naturally infected in the wild; however without the use of bioluminescence to visualise *M.*  
256 *marinum*, the easy identification of infected embryos would not be possible. We observed that the  
257 proportion of embryos which became infected varied depending on the housing conditions. Initially  
258 embryos were housed in groups of 50 for infection but this resulted in a low proportion of  
259 measurable infections. When we increased the number to 300, the proportion of embryos with a  
260 measurable infection rose to approximately 40%. We speculate that this is due to the increased  
261 motion of the fish in the media allowing for greater mixing. Another possibility is that *M. marinum*  
262 may phenotypically change as it travels through the embryo gut, making it more infectious. Such  
263 hyperinfectivity has been reported for *Vibrio cholera* and *Citrobacter rodentium*<sup>46-48</sup>  
264 As several bioluminescent reporter systems exist, we wanted to establish which one would give the  
265 best results for this assay. We compared mycobacterial optimised bacterial luciferase (lux) and the  
266 codon-optimised red shifted firefly luciferase (RTluc)<sup>49</sup>. The bacterial lux construct contains all the  
267 required genes to make both the substrate and the catalytic enzyme to produce light. In the case of  
268 the firefly luciferase, the substrate has to be added exogenously. While *M. marinum* tagged with the  
269 firefly luciferase did produce higher levels of light, this was not enough to compensate for the  
270 increased cost to the assay, both in terms of expense and time as a result of having to add the  
271 exogenous substrate. We chose to use *M. marinum* tagged with bacterial lux through-out to produce  
272 as streamlined and economic an assay as possible.

273 We investigated whether the assay could be accelerated by using pronase treatment to  
274 dechorionate embryos. While pronase treatment did reduce the time required to prepare embryos,  
275 more bacteria were needed to establish an infection compared to the dose needed to infect  
276 manually dechorionated embryos. Similarly, the proportion of infected embryos with visible  
277 bioluminescence was also reduced. One possible explanation for this difference could be that

278 pronase treatment may result in an increased inflammatory reaction within the embryos, resulting in  
279 greater initial killing of the bacteria.

280 The optimised natural infection assay protocol we have established requires manual dechorination  
281 of zebrafish embryos two days post fertilisation, followed by bathing with  $1 \times 10^7$  cfu ml<sup>-1</sup> *M. marinum*  
282 for 4 days. Embryos are then removed from the infected media, washed and placed in fresh media  
283 and kept for a further overnight to allow for the clearance of transiently colonising bacteria. After a  
284 further wash, embryos can be housed within individual wells of a clear bottom, black 96 well plate  
285 for measurement of infection dynamics using bioluminescence. We investigated whether the  
286 optimised natural infection assay could be applied to the testing of anti-mycobacterial compounds.  
287 Four of the six compounds we tested reduced the luminescence from infected embryos to below  
288 background levels. When the same compounds were tested in the caudal vein-injection model,  
289 which uses embryo survival as an indicator of bacterial clearance, three of the four compounds were  
290 also identified as being effective.

291 Overall this study has demonstrated that it is possible to carry out high throughput in vivo drug  
292 screening in the zebrafish model using embryos naturally infected with bioluminescent *M. marinum*  
293 *M. marinum*. Natural infection is quicker than injection and requires less expertise. Interestingly, not all of the  
294 injected embryos had detectable light levels and with the fluorescently tagged *M. marinum* up to  
295 90% of the naturally infected embryos had visible signs of infection (data not shown). Embryos can  
296 be screened in 96 well plates and drug efficacy rapidly identified over the course of 10 days. Through  
297 the use of a luminometer with a plate stacker this process can be semi-automated to reduce the  
298 hands on time. While this is moderately slower than previously reported automated robotic systems,  
299 it is also a fraction of the cost. The result is an assay that can be carried out by a wide variety of  
300 laboratories for minimal cost and without high levels of zebrafish expertise. Widening participation  
301 in TB research and pre-clinical drug discovery in this way should accelerate the progress towards  
302 new and better treatments for TB and other neglected mycobacterial infections.

303

304

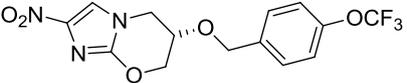
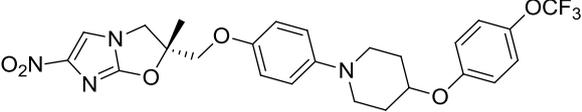
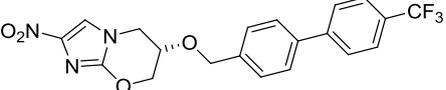
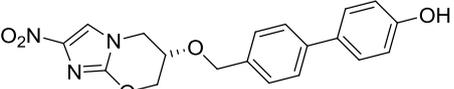
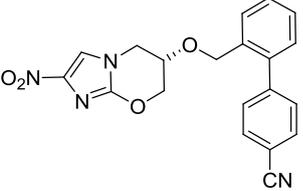
Strain/plasmid	Description	Reference
<i>M. marinum</i> ATCC BAA-535	Wildtype isolate M	
<i>M. marinum</i> BSG100	ATCC BAA-535 containing pMV306G13FFlucRT chromosomally integrated, Km resistant.	This study
<i>M. marinum</i> BSG101	ATCC BAA-535 containing pMV306G13LuxABCDE chromosomally integrated, Km resistant.	This study
<i>M. marinum</i> BSG102	ATCC BAA-535 expressing pTEC27, Hyg resistant.	This study
<i>M. marinum</i> BSG103	BSG101 expressing pTEC27, Km and Hyg resistant.	This study
<i>M. tuberculosis</i> BSG001	<i>M. tuberculosis</i> ATCC (H37Rv) containing MV306hsp + LuxAB + G13 + CDE chromosomally integrated, Km resistant.	50
pMV306G13FFlucRT	Mycobacterial integrating vector containing a modified firefly luciferase gene optimised for use in Mycobacteria, Km resistant.	49
pMV306G13LuxABCDE	Mycobacterial integrating vector containing the <i>lux</i> operon from <i>Photobacterium luminescens</i> optimised for use in Mycobacteria, Km resistant.	49
pTEC27	Mycobacterial plasmid containing the red fluorescent protein tdTomato, hyg resistant.	13

305 **Table 1: Strains and plasmids used in this study**

306 Key: Km, kanamycin; hyg, hygromycin.

307

308

Compound ID	Structure	In vitro MIC	
		<i>M. tuberculosis</i>	<i>M. marinum</i>
Pretomanid <sup>39</sup>		0.3125 μM	>10 μM
Delamanid <sup>39</sup>		0.078 μM	0.625 μM
SN30488 <sup>40</sup>		0.039 μM	>10 μM
SN30527 <sup>40</sup>		0.625 μM	10 μM
SN30982 <sup>40</sup>		10 μM	>10 μM

309 **Table 2. Novel compounds used in drug treatment assays and in vitro MIC values for *M.***

310 ***tuberculosis* BSG001 and *M. marinum* BSG101**

311 Key: MIC, minimum inhibitory concentration.

312

313

Compound ID	In vitro MTB	In vitro MM	Zebrafish MM (natural infection)	Zebrafish MM (caudal vein injection)
Pretomanid	A	N	A	A
Delamanid	A	A	A	A
SN30488	A	N	A	NT
SN30527	A	A	A	NT
SN30982	N	N	N	N
Rifampicin	A	A	A	N

314

315 **Table 3. Comparison of efficacy of treatments across in vitro and in vivo models used in this study.**

316 Key: MTB, *M. tuberculosis* BSG001; MM, *M. marinum* BSG101; A, active; N, not active; NT, not

317 tested.

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320

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325

## 326 Transparency statement

327 No conflicts of interest to declare.

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445 **Figure Legends**

446 **Figure 1: Bioluminescence can be used to monitor zebrafish embryos naturally infected with**  
447 **tagged strains of *M. marinum*.** A comparison of the bioluminescence (given as relative light units  
448 [RLU]) from zebrafish embryos naturally infected with *M. marinum* M expressing either a red-shifted  
449 firefly luciferase (BSG100) or bacterial luciferase (BSG101). Similar light levels were observed with  
450 the two constructs while uninfected embryos remained at background levels (indicated by black  
451 dashed line). Bioluminescence is presented as box-whisker plots of RLUs from 20-30 embryos  
452 measured over a seven day period. The edges of the boxes represent the 25th and 75th quartiles,  
453 the solid line represents the median, and the whiskers are the minimum and maximum values. One  
454 representative experiment is shown.

455

456 **Figure 2: Natural exposure of zebrafish embryos to *M. marinum* results in transient colonisation of**  
457 **the gut and infection of the developing gills and lower jaw.** Embryos were exposed to *M. marinum*  
458 expressing a red fluorescent reporter (BSG102) and bacterial location identified by fluorescence  
459 microscopy. (A) Colonisation of the developing gills and lower jaw after 4 days infection. (B)  
460 Transient colonisation of the digestive tract. (C) After 5 days, colonisation is localised to the head  
461 region, with the digestive tract no longer colonised. Representative embryos are shown.

462

463 **Figure 3: An infectious dose of  $10^7$  cfu bioluminescent *M. marinum* per ml of exposed medium is**  
464 **required to produce infected embryos that can be detected by luminometry.** A comparison of the  
465 bioluminescence (given as relative light units [RLU]) from zebrafish embryos naturally infected with  
466 different doses (from  $10^4$  to  $10^7$  colony forming units [cfu]  $\text{ml}^{-1}$ ) of *M. marinum* M expressing  
467 bacterial luciferase (BSG101). Black dashed line indicates limits of detection. Bioluminescence is  
468 presented as box-whisker plots of RLUs from 20-30 embryos measured over a 12 day period. The

469 edges of the boxes represent the 25th and 75th quartiles, the solid line represents the median, and  
470 the whiskers are the minimum and maximum values. One representative experiment is shown.

471

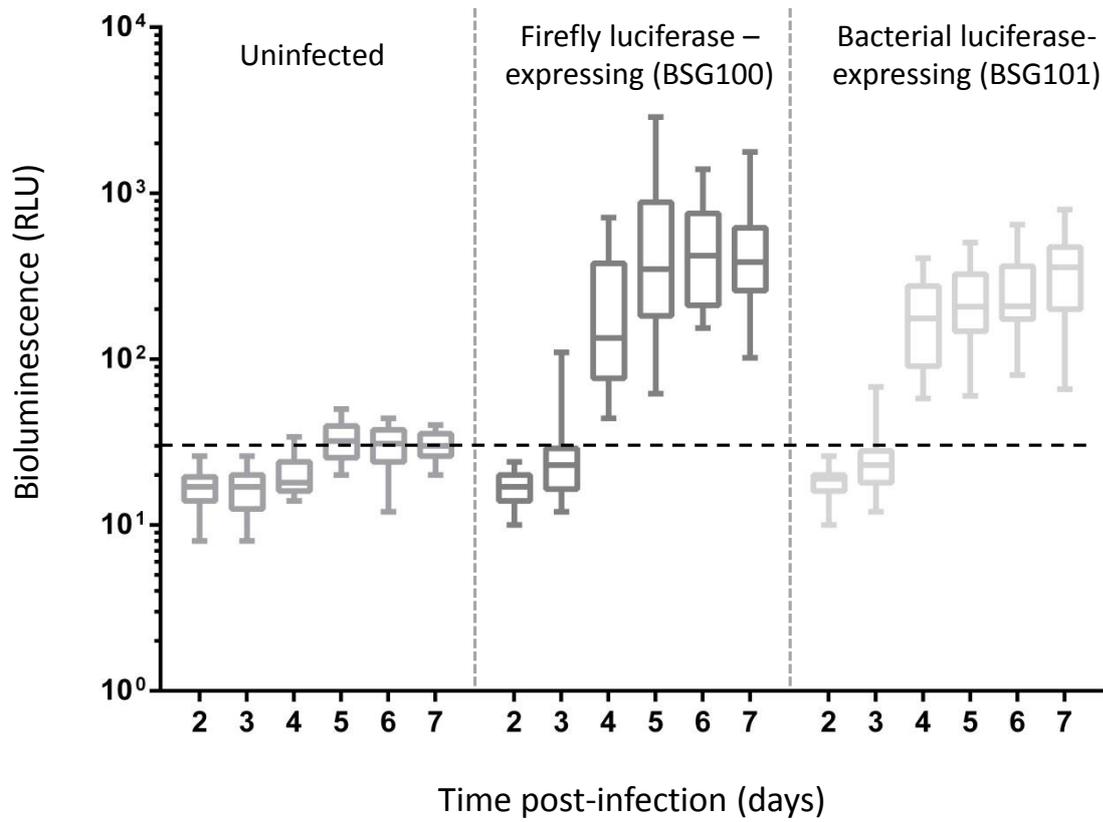
472 **Figure 4. Treatment of *M. marinum* BSG101-infected embryos with Pretomanid, Delamanid,**  
473 **SN30488, SN30527, SN30982 and rifampicin.** Drug efficacy was monitored by changes in  
474 bioluminescence (given as relative light units [RLU]) from individual naturally infected embryos  
475 (n=20-30) immediately prior to and 3 days after treatment (A). Black dashed line indicates limits of  
476 detection of luminometer. Data did not pass the D'Agostino & Pearson normality test so before and  
477 after treatment groups were compared using the Kruskal Wallis test with Dunn's post-hoc analysis.  
478 Those treatments resulting in a significant decrease in bioluminescence are shown. One  
479 representative experiment out of 3 is shown. For embryos infected by caudal vein injection (B), after  
480 injection embryos were placed directly into media containing compounds and drug efficacy was  
481 monitored by changes in survival over ten days. One representative experiment out of 2 is shown.

482

483 **Figure 5. In vitro treatment of *M. marinum* BSG101 and *M. tuberculosis* BSG001 with Pretomanid,**  
484 **Delamanid, SN30488, SN30527, SN30982 and rifampicin.** Drug efficacy was monitored by changes  
485 in bioluminescence (given as relative light units [RLU]) immediately prior to and 3 days after  
486 treatment for *M. marinum* BSG101 (A) or for *M. tuberculosis* BSG001 (B). Data passed the D'Agostino  
487 & Pearson normality test so before and after treatment groups were compared using ANOVA with  
488 Bonferroni's post-hoc analysis. Those treatments resulting in a significant increase in  
489 bioluminescence are shown. Data represents experiments performed on three separate occasions.  
490 (Dotted line indicates background level of light detection).

491

Fig 1



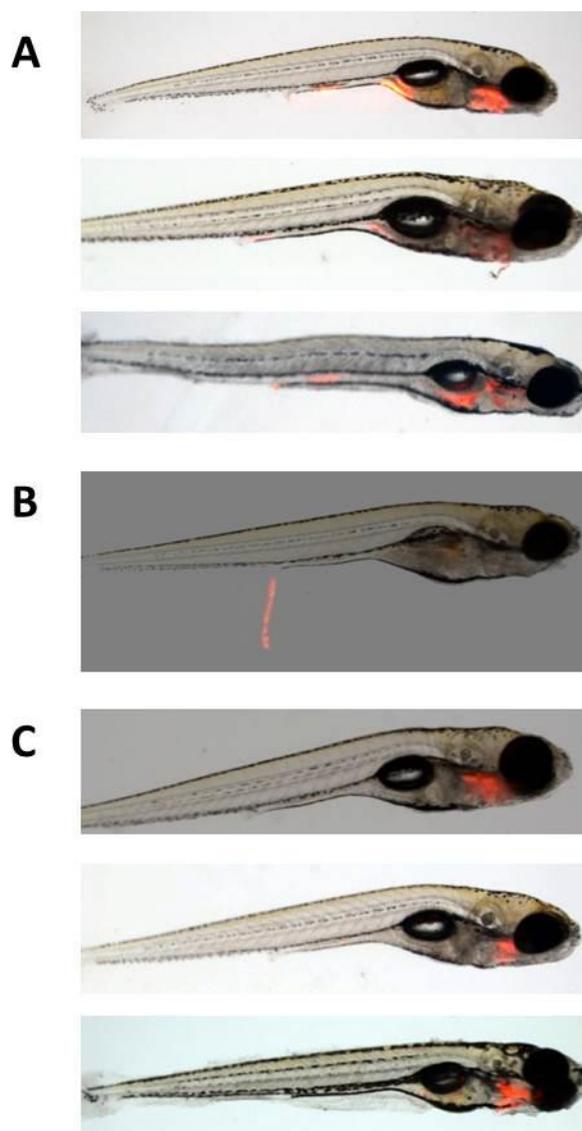
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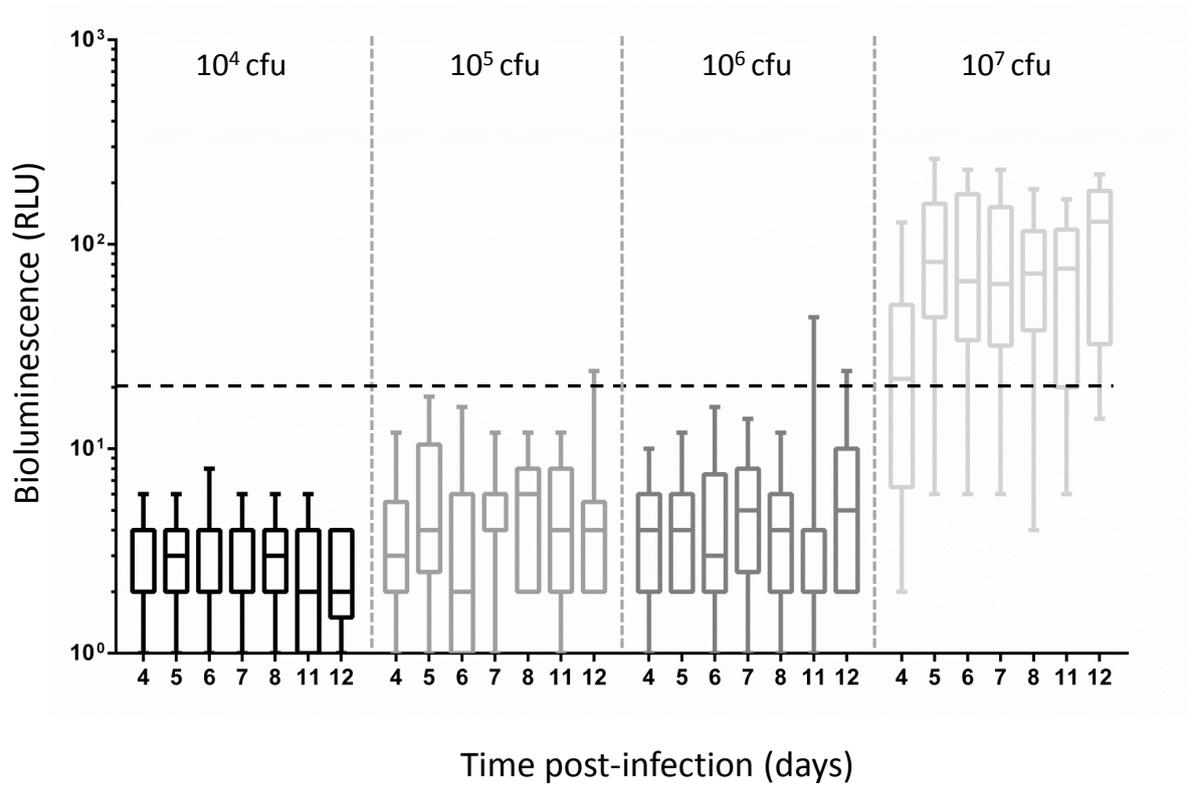
Fig 2



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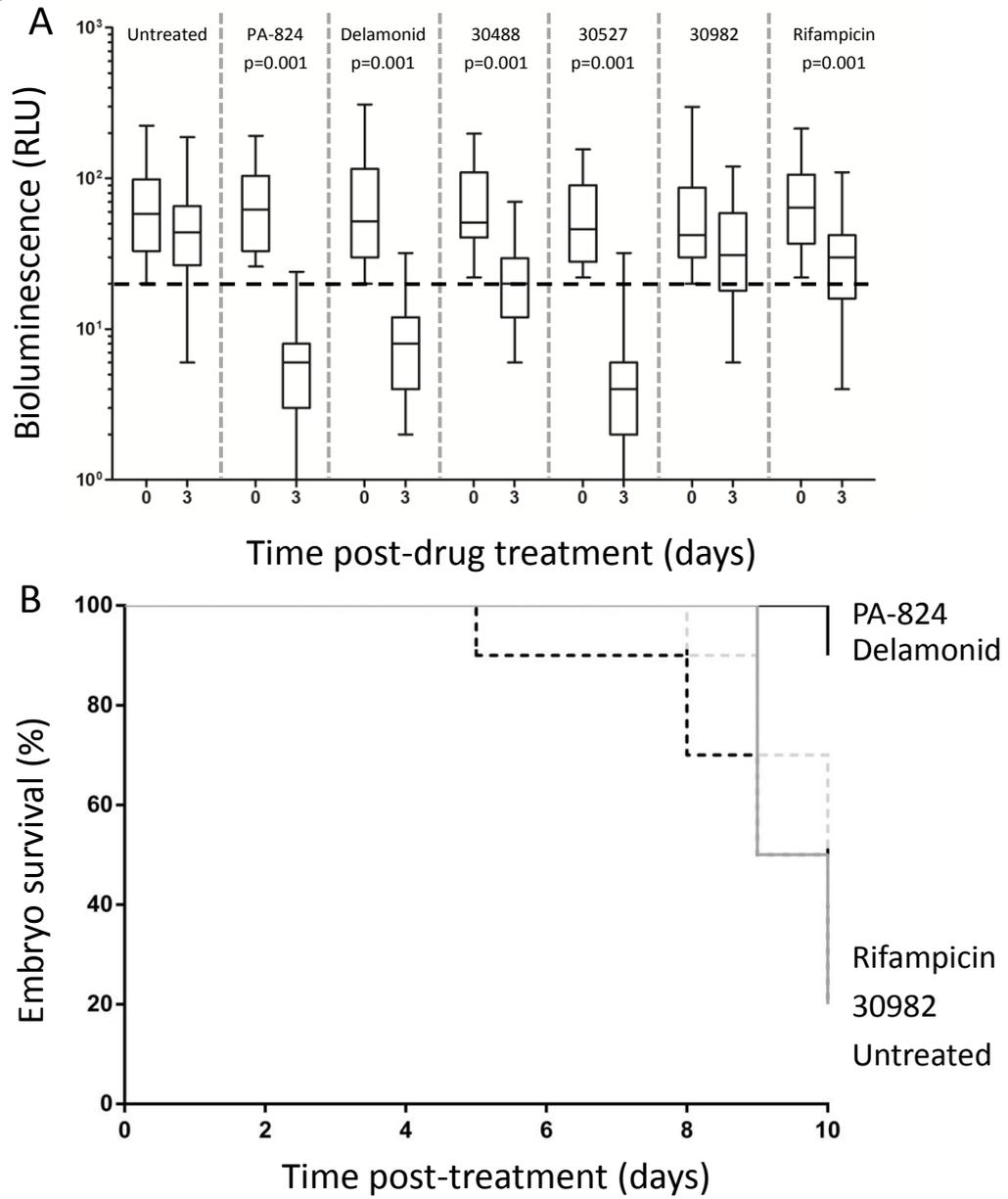
Fig 3



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Fig 4



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Fig 5

