Screening of anti-mycobacterial compounds in a naturally infected zebrafish embryo model

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Synopsis

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

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

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

Introduction

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

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

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[27, 28]
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 [28-30]. 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[28, 31-33].

77 Zebrafish and other fish readily become naturally infected with *M. marinum* present in their
78 environment[34]. 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[26]. 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

Materials and Methods

Bacterial strains and plasmids

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

In vitro drug testing

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

Fish husbandry

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

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

Natural infection of zebrafish embryos

M. marinum strains were grown to mid log phase (OD_{600} between 0.8 and 2) without antibiotic selection, washed once in E3 before being adjusted to an OD_{600} of 1. The approximate concentration should be around $5x10^8$ cfu ml^{-1}; this was confirmed by retrospectively plating inocula onto 7H11 supplemented with 10% OADC and 0.5% glycerol. Zebrafish embryos at 2 days post-fertilisation (dpf) were dechorionated, either manually or using pronase, as previously described[36]. Groups of embryos (50-300) were then placed in 9mm petri dishes containing 25ml of E3 supplemented with varying concentrations of *M. marinum* (by varying the volume of adjusted bacteria) and incubated for 4 days at 28°C. After infection, any non-internalised bacteria were removed by gently washing embryos four times with fresh E3 in groups of 10 in separate wells of a 24 well tissue culture plates (BD Falcon) using gentle aspiration of the media. Embryos were left in the 24 well plate overnight to remove transient bacteria and then rinsed 4 times in E3 to remove the transient population. Embryos were then individually placed into the wells of a clear-bottomed black 96 well microtitre plate (Nunc) with 100µl of E3 and prepared for drug treatment.

Injection of zebrafish embryos

M. marinum strains were prepared as above. Zebrafish embryos 2dpf were manually de-chorionated, anaesthetised using 0.168 mg ml^{-1} tricaine (Sigma-Aldrich) in E3 medium[37] and infected by microinjection into the caudal vein as previously described[38].

Measurement of bioluminescence from zebrafish embryos

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

Drug testing in zebrafish embryos

Infected zebrafish embryo were read on a luminometer after removal of transient bacteria and detectably infected embryos (RLU >20) were randomly distributed within drug treatment groups. Drugs were made up to 100x working concentration in an appropriate solvent. They were diluted 1:10 in E3 and 10µl of diluted drug was placed into each well of the 96 well plate containing 100µl of E3. The final concentration of the test compounds was 10µM. E3 was used as a no treatment control. Embryos were treated at 5 days post infection and incubated at 28°C during treatment.

Microscopy

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

Statistics

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

Results

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

To determine if zebrafish embryos infected with bioluminescently labelled *M. marinum* could be monitored using a luminometer, we incubated embryos (n=300) aged 1 and 2 dpf in E3 containing $1x10^7$ cfu ml^{-1} of *M. marinum* tagged either with a modified firefly luciferase (designated *M. marinum* BSG100) or with a modified bacterial luciferase (designated *M. marinum* BSG101). At different time points, we removed the embryos from the *M. marinum*-containing media, washed them in fresh media and measured light. As the firefly luciferase reaction requires luciferin as a substrate, we added this exogenously (30 µg ml^{-1}) when embryos were placed into the 96 well plates. Bioluminescence from infected embryos did not rise above background levels until 4 days post infection, with comparable levels of light emitted from BSG100 and BSG101 infected embryos (Fig. 1). Natural infection of embryos with *M. marinum* did not result in any premature deaths when compared to the uninfected group. While embryos infected with *M. marinum* labelled with the firefly luciferase (BSG100) produced more light (median maximum value of 709 relative light units [RLU] (ranging from 328 to 2872)) compared to the bacterial luciferase tagged strain (BSG101) (median maximum value of 446 RLU (ranging from 122 to 796)), the exogenous addition of luciferin increased the time required to carry out the assay and the expense of the technique. We therefore selected bacterial-luciferase tagged *M. marinum* M (BSG101) for further study.

Natural infection results in gill colonisation and transient gut colonisation

We investigated the nature and location of natural *M. marinum* infection of embryos using a strain of *M. marinum* expressing the red fluorescent protein tdTomato[13] (designated BSG102). We incubated the embryos with BSG102 for 4 days and then determined the location of the infecting 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 $1x10^4$ to $1x10^7$cfu ml^{-1} and followed bioluminescence over 12 days using a luminometer. A dose of
190 $1x10^7$cfu ml^{-1} 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 dechorionation (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 dechorionation of embryos 2 dpf, 2) immersion in media containing
203 $1x10^7$cfu ml^{-1} *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[39]. The experimental compounds SN30488,
212 SN30527 and SN30982[40] 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 (Delamonid,
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

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

Discussion

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

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

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

252 open-ended tube by 4 dpf[45]. 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*[46-48]

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)[49]. 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 $1x10^7$ cfu ml^{-1} *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. 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

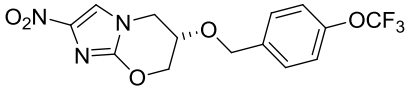
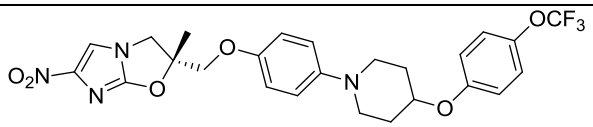
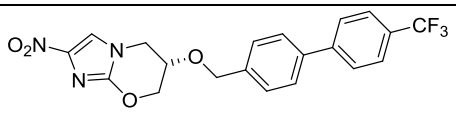
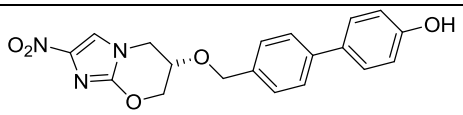
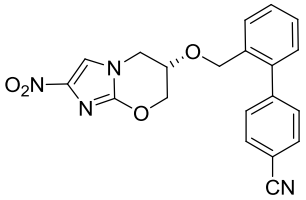
Strain/plasmid	Description	Reference
M. marinum ATCC BAA-535	Wildtype isolate M	
M. marinum BSG100	ATCC BAA-535 containing pMV306G13FFlucRT chromosomally integrated, Km resistant.	This study
M. marinum BSG101	ATCC BAA-535 containing pMV306G13LuxABCDE chromosomally integrated, Km resistant.	This study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

		In vitro MIC	
Compound ID	Structure	*M. tuberculosis*	*M. marinum*
Pretomanid[39]		0.3125 µM	>10 µM
Delamanid[39]		0.078 µM	0.625 µM
SN30488[40]		0.039 µM	>10 µM
SN30527[40]		0.625 µM	10 µM
SN30982[40]		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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Transparency statement

No conflicts of interest to declare.

References

1. WHO. Global tuberculosis report 2015. Geneva, Switzerland, 2015.
2. Shah NS, Wright A, Bai GH et al. Worldwide emergence of extensively drug-resistant tuberculosis. *Emerg Infect Dis* 2007; **13**: 380-7.
3. Gandhi NR, Nunn P, Dheda K et al. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. *The Lancet*; **375**: 1830-43.
4. Getahun H, Gunneberg C, Granich R et al. HIV infection-associated tuberculosis: the epidemiology and the response. *Clin Infect Dis* 2010; **50 Suppl 3**: S201-7.
5. Forbes L, Ebsworth-Mojica K, DiDone L et al. A High Throughput Screening Assay for Anti-Mycobacterial Small Molecules Based on Adenylate Kinase Release as a Reporter of Cell Lysis. *PLoS One* 2015; **10**: e0129234.
6. Altaf M, Miller CH, Bellows DS et al. Evaluation of the Mycobacterium smegmatis and BCG models for the discovery of Mycobacterium tuberculosis inhibitors. *Tuberculosis (Edinb)* 2010; **90**: 333-7.
7. Xu S, Pei L, Li D et al. Synthesis and antimycobacterial evaluation of natural oridonin and its enmein-type derivatives. *Fitoterapia* 2014; **99**: 300-6.
8. Abuhammad A, Fullam E, Bhakta S et al. Exploration of piperidinols as potential antitubercular agents. *Molecules* 2014; **19**: 16274-90.
9. Tobin DM, Ramakrishnan L. Comparative pathogenesis of Mycobacterium marinum and Mycobacterium tuberculosis. *Cell Microbiol* 2008; **10**: 1027-39.
10. Ramakrishnan L. Images in clinical medicine. Mycobacterium marinum infection of the hand. *N Engl J Med* 1997; **337**: 612.
11. Bonamonte D, De Vito D, Vestita M et al. Aquarium-borne Mycobacterium marinum skin infection. Report of 15 cases and review of the literature. *Eur J Dermatol* 2013; **23**: 510-6.

12. Ramakrishnan L. Looking within the zebrafish to understand the tuberculous granuloma. *Adv Exp Med Biol* 2013; **783**: 251-66.
13. Takaki K, Davis JM, Winglee K et al. Evaluation of the pathogenesis and treatment of Mycobacterium marinum infection in zebrafish. *Nat Protocols* 2013; **8**: 1114-24.
14. Singh SK, Sethi S, Aravamudhan S et al. Proteome mapping of adult zebrafish marrow neutrophils reveals partial cross species conservation to human peripheral neutrophils. *PLoS One* 2013; **8**: e73998.
15. Rauta PR, Nayak B, Das S. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunol Lett* 2012; **148**: 23-33.
16. van der Vaart M, Spaink HP, Meijer AH. Pathogen recognition and activation of the innate immune response in zebrafish. *Adv Hematol* 2012; **2012**: 159807.
17. Trede NS, Langenau DM, Traver D et al. The use of zebrafish to understand immunity. *Immunity* 2004; **20**: 367-79.
18. Ridges S, Heaton WL, Joshi D et al. Zebrafish screen identifies novel compound with selective toxicity against leukemia. *Blood* 2012; **119**: 5621-31.
19. Tan JL, Zon LI. Chemical screening in zebrafish for novel biological and therapeutic discovery. *Methods Cell Biol* 2011; **105**: 493-516.
20. Prajsnar TK, Hamilton R, Garcia-Lara J et al. A privileged intraphagocyte niche is responsible for disseminated infection of Staphylococcus aureus in a zebrafish model. *Cell Microbiol* 2012; **14**: 1600-19.
21. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 2007; **8**: 353-67.
22. Meijer AH, Spaink HP. Host-pathogen interactions made transparent with the zebrafish model. *Curr Drug Targets* 2011; **12**: 1000-17.
23. Mostowy S, Boucontet L, Mazon Moya MJ et al. The Zebrafish as a New Model for the In Vivo Study of Shigella flexneri Interaction with Phagocytes and Bacterial Autophagy. *PLoS Pathog* 2013; **9**: e1003588.
24. Cui C, Benard EL, Kanwal Z et al. Infectious disease modeling and innate immune function in zebrafish embryos. *Methods Cell Biol* 2011; **105**: 273-308.
25. Prouty MG, Correa NE, Barker LP et al. Zebrafish-Mycobacterium marinum model for mycobacterial pathogenesis. *FEMS Microbiology Letters* 2003; **225**: 177-82.
26. Swaim LE, Connolly LE, Volkman HE et al. Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect Immun* 2006; **74**: 6108-17.
27. Waidmann MS, Bleichrodt FS, Laslo T et al. Bacterial luciferase reporters: the Swiss army knife of molecular biology. *Bioeng Bugs* 2011; **2**: 8-16.
28. Andreu N, Fletcher T, Krishnan N et al. Rapid measurement of antituberculosis drug activity in vitro and in macrophages using bioluminescence. *J Antimicrob Chemother* 2012; **67**: 404-14.
29. Corr SC, Li Y, Riedel CU et al. Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. *Proceedings of the National Academy of Sciences of the United States of America* 2007; **104**: 7617-21.
30. Bron PA, Monk IR, Corr SC et al. Novel luciferase reporter system for in vitro and organ-specific monitoring of differential gene expression in Listeria monocytogenes. *Appl Environ Microbiol* 2006; **72**: 2876-84.
31. Andreu N, Zelmer A, Sampson SL et al. Rapid in vivo assessment of drug efficacy against Mycobacterium tuberculosis using an improved firefly luciferase. *J Antimicrob Chemother* 2013; **68**: 2118-27.
32. Xiong YQ, Willard J, Kadurugamuwa JL et al. Real-time in vivo bioluminescent imaging for evaluating the efficacy of antibiotics in a rat Staphylococcus aureus endocarditis model. *Antimicrob Agents Chemother* 2005; **49**: 380-7.

33. Kadurugamuwa JL, Modi K, Yu J et al. Noninvasive monitoring of pneumococcal meningitis and evaluation of treatment efficacy in an experimental mouse model. *Mol Imaging* 2005; **4**: 137-42.
34. Ramsay JM, Watral V, Schreck CB et al. Husbandry stress exacerbates mycobacterial infections in adult zebrafish, Danio rerio (Hamilton). *J Fish Dis* 2009; **32**: 931-41.
35. Goude R, Parish T. Electroporation of mycobacteria. *Methods Mol Biol* 2009; **465**: 203-15.
36. Kim DH, Sun Y, Yun S et al. Mechanical property characterization of the zebrafish embryo chorion. *Conf Proc IEEE Eng Med Biol Soc* 2004; **7**: 5061-4.
37. Westerfield M. *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*: Univ. of Oregon Press, Eugene, 2000.
38. Davis JM, Ramakrishnan L. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 2009; **136**: 37-49.
39. Olaru ID, von Groote-Bidlingmaier F, Heyckendorf J et al. Novel drugs against tuberculosis: a clinician's perspective. *Eur Respir J* 2015; **45**: 1119-31.
40. Palmer BD, Thompson AM, Sutherland HS et al. Synthesis and structure-activity studies of biphenyl analogues of the tuberculosis drug (6S)-2-nitro-6-{[4-(trifluoromethoxy)benzyl]oxy}-6,7-dihydro-5H-imidazo[2,1-b][1, 3]oxazine (PA-824). *J Med Chem* 2010; **53**: 282-94.
41. Barker LP, Lien BA, Brun OS et al. A Mycobacterium marinum zone of inhibition assay as a method for screening potential antimycobacterial compounds from marine extracts. *Planta Med* 2007; **73**: 559-63.
42. Kotapalli SS, Nallam SSA, Nadella L et al. Identification of New Molecular Entities (NMEs) as Potential Leads against Tuberculosis from Open Source Compound Repository. *PLoS One* 2015; **10**: e0144018.
43. Sridevi JP, Anantaraju HS, Kulkarni P et al. Optimization and validation of Mycobacterium marinum-induced adult zebrafish model for evaluation of oral anti-tuberculosis drugs. *International Journal of Mycobacteriology* 2014; **3**: 259-67.
44. Carvalho R, de Sonneville J, Stockhammer OW et al. A high-throughput screen for tuberculosis progression. *PLoS One* 2011; **6**: e16779.
45. Bates JM, Mittge E, Kuhlman J et al. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Developmental Biology* 2006; **297**: 374-86.
46. Alam A, Larocque RC, Harris JB et al. Hyperinfectivity of human-passaged Vibrio cholerae can be modeled by growth in the infant mouse. *Infect Immun* 2005; **73**: 6674-9.
47. Merrell DS, Butler SM, Qadri F et al. Host-induced epidemic spread of the cholera bacterium. *Nature* 2002; **417**: 642-5.
48. Wiles S, Dougan G, Frankel G. Emergence of a 'hyperinfectious' bacterial state after passage of Citrobacter rodentium through the host gastrointestinal tract. *Cell Microbiol* 2005; **7**: 1163-72.
49. Andreu N, Zelmer A, Fletcher T et al. Optimisation of bioluminescent reporters for use with mycobacteria. *PLoS One* 2010; **5**: e10777.
50. Wang J, Pearce AN, Chan ST et al. Biologically Active Acetylenic Amino Alcohol and N-Hydroxylated 1,2,3,4-Tetrahydro-beta-carboline Constituents of the New Zealand Ascidian Pseudodistoma opacum. *J Nat Prod* 2015.

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] 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

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

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

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

Fig 1

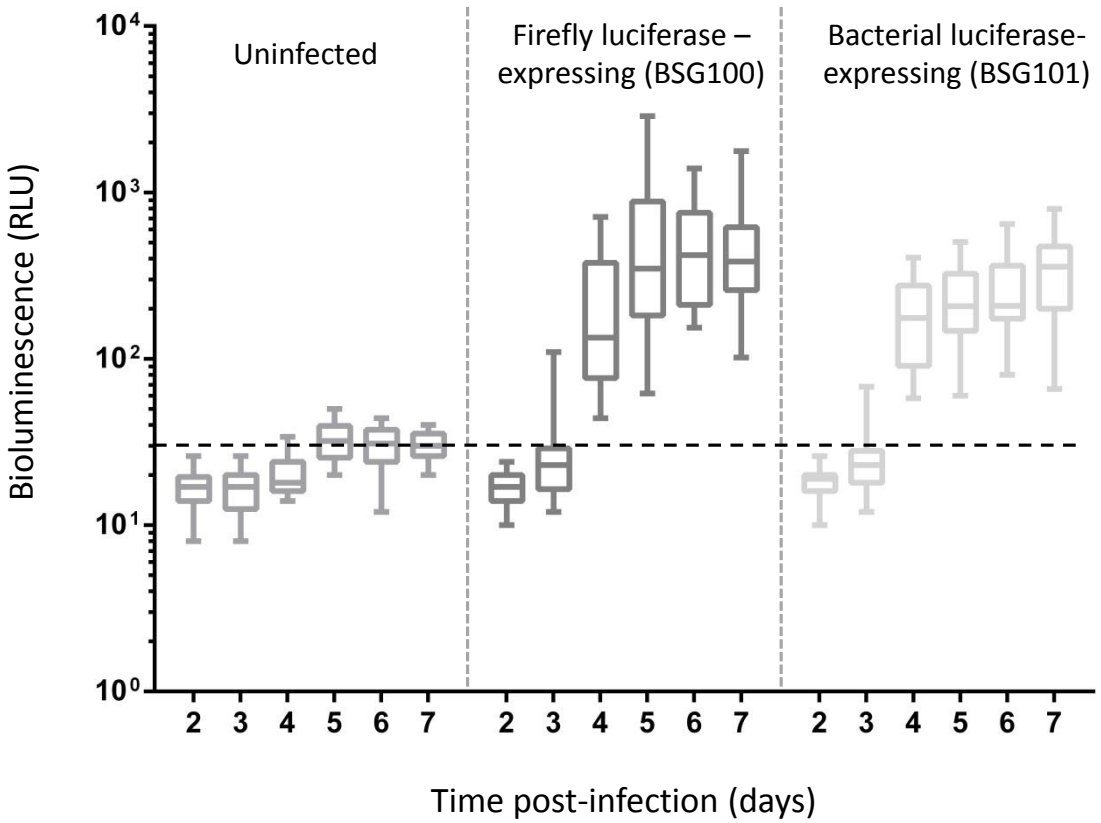

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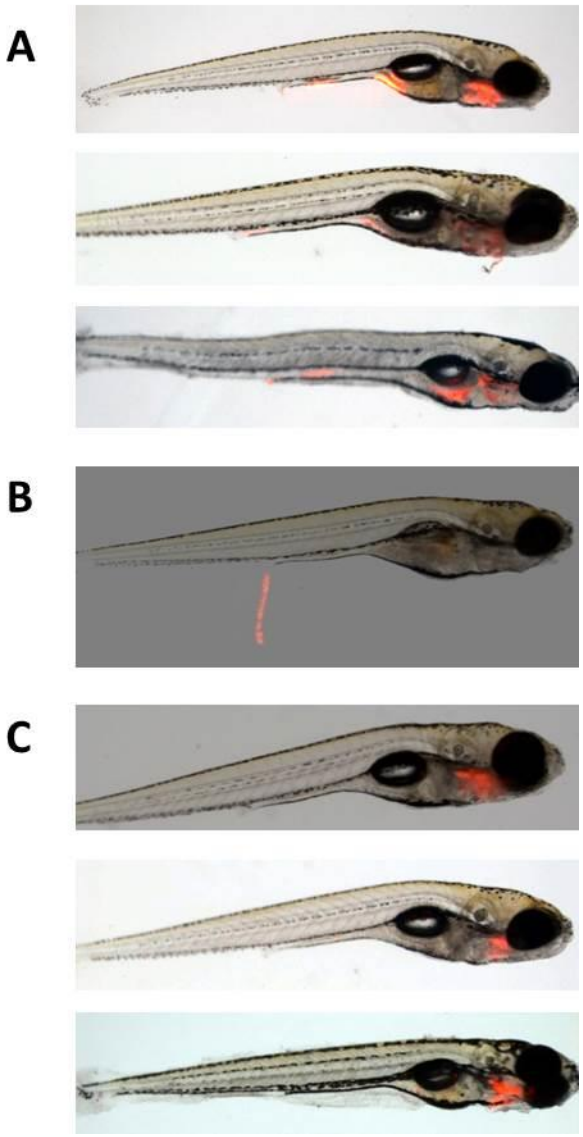
Fig 2



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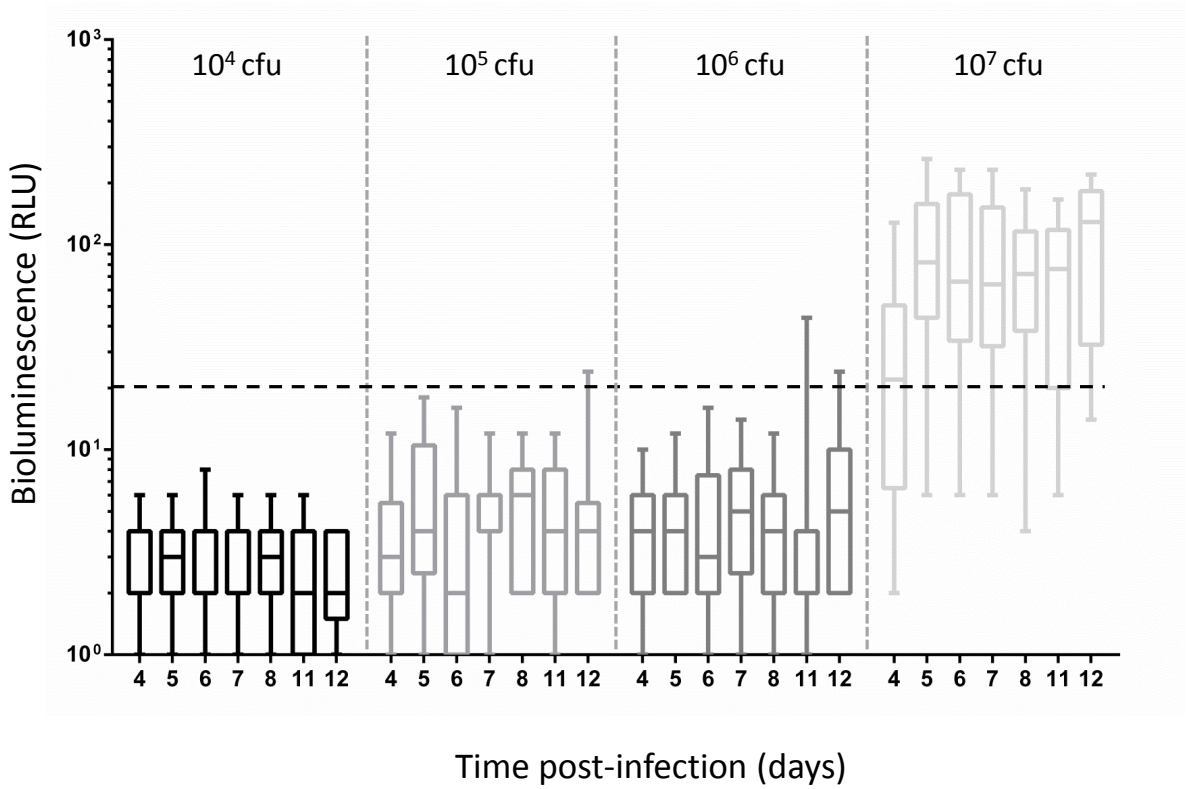

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Fig 4

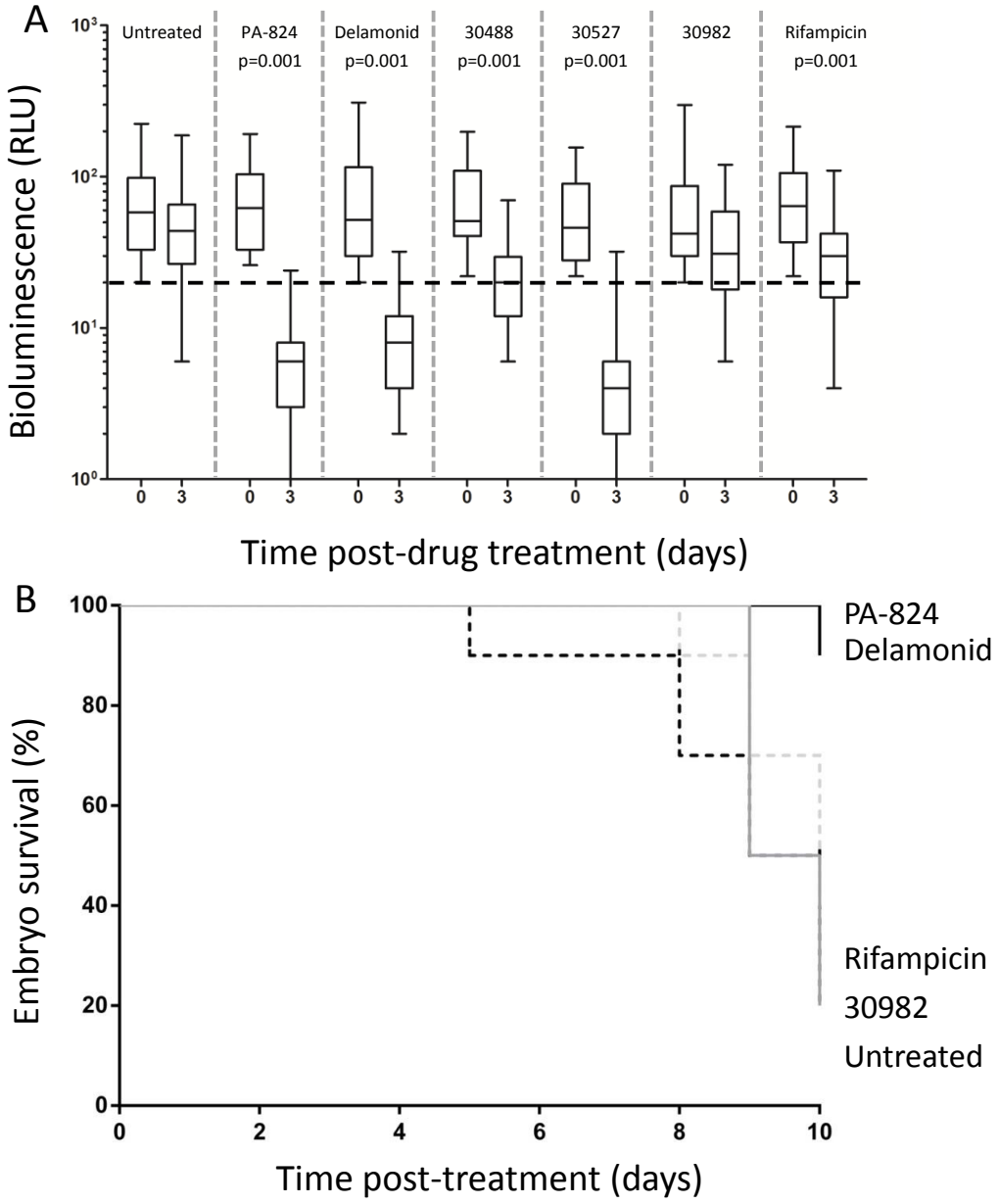

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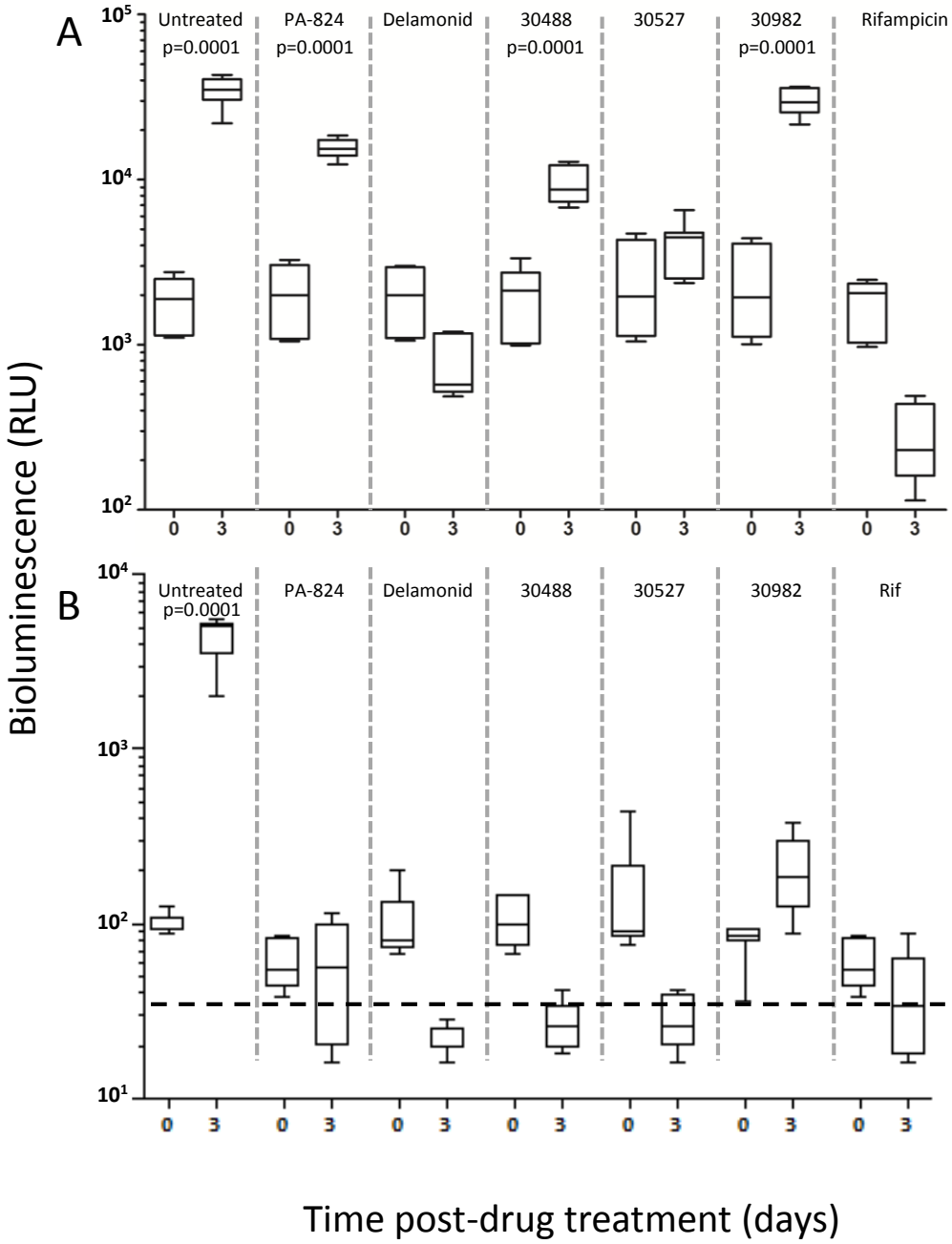
Fig 5



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