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► **To cite this version:**

Matt D Johansen, Claire Hamela, Yi Ding, Laurent Kremer. Zebrafish Models for Drug Discovery and Therapeutic Validation against Non-Tuberculous Mycobacteria. Cold Spring Harbor Perspectives in Medicine, In press, <10.1101/cshperspect.a041832>. <hal-05034930>

HAL Id: hal-05034930

<https://hal.science/hal-05034930v1>

Submitted on 15 Apr 2025

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Zebrafish models for drug discovery and therapeutic validation against non-tuberculous mycobacteria

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Keywords: Non-tuberculous mycobacteria, zebrafish, infection, drugs, host-directed therapy.

Running title: Zebrafish to assess anti-NTM treatments

ABSTRACT

The incidence of non-tuberculous mycobacteria (NTM) is increasing globally, often surpassing the incidence of new tuberculosis cases in developed countries. Most NTM are environmental organisms, however, there are a number of opportunistic and pathogenic species that can cause severe infections in animals and humans. Many NTM are intrinsically resistant to anti-tuberculosis therapies and are incredibly difficult to treat, resulting in poor treatment outcomes for these patients. Recent advances in preclinical animal models such as the zebrafish models have led to the discovery of highly active antimicrobial and host-directed therapies targeting NTM infections that can be applied to treat human infections. Here, we summarize recent progress and technological advancements in the discovery and development of antimicrobial drugs and host-directed therapies that have been applied to NTM zebrafish infection models. We highlight the future directions for this increasingly applicable animal model for the discovery of next-generation therapies to treat NTM diseases.

INTRODUCTION

The genus *Mycobacterium* can be broadly split into two different groups: Tuberculosis (TB)-causing mycobacteria, and non-tuberculous mycobacteria (NTM). Interestingly, *Mycobacterium leprae* (the causative agent of Leprosy) is genetically distinct to all other *Mycobacterium* spp. and is often placed into an independent genetic clade. There are over 200 different NTM that have been identified to date that are classified according to their growth rate as rapid-growing and slow-growing species (Tortoli 2014; Tortoli *et al.* 2017). Of these identified NTM species, >95% are either environmental or saprophytic organisms that are non-pathogenic and pose no risk to animal or human health (Johansen *et al.* 2020b). However, there are a number of pathogenic and opportunistic NTM species that are responsible for significant infectious diseases such as *Mycobacterium abscessus*, *Mycobacterium avium* and *Mycobacterium marinum* (**Figure 1A**). These pathogenic NTM infections affect a wide range of host tissues and organs, including the pulmonary system, skin and soft tissue, lymph nodes and in severe cases can cause disseminated disease manifestations with multi-organ involvement (Baldwin *et al.* 2019). Currently available treatments for NTM infections are extensive, requiring prolonged chemotherapy involving macrolides and aminoglycosides (Clarithromycin (CLR) and Amikacin (AMK), respectively) (Nick *et al.* 2021; Kumar *et al.* 2022). The majority of NTM species are intrinsically resistant to many frontline antimicrobials used to treat TB such as Isoniazid and Rifampicin, and are often

misdiagnosed as multi-drug resistant TB infections (Wu *et al.* 2018; Raju *et al.* 2016). Furthermore, pathogenic species such as *M. abscessus* possess a high degree of innate and acquired mechanisms of resistance and are able to overcome antimicrobial treatment regimens (Nash *et al.* 2009; Luthra *et al.* 2018), resulting in cure rates as low as 45% depending on the tissue site and NTM species responsible. Treatment failure in NTM infections can lead to chronic and lifelong infections, and can result in patient death (Nick *et al.* 2021; Kumar *et al.* 2022). As such, there is an urgent need to identify novel therapeutics that are highly active against opportunistic and pathogenic NTM species. In the identification of novel and leading drug candidates for the treatment of NTM infections, preclinical animal models represent the cornerstone of drug discovery platforms for human safety and efficacy trials. In recent years, the zebrafish model has been used extensively to uncover novel pathogenic traits of several NTM species (**Figure 1A**), and to validate leading preclinical drug candidates for the treatment of multiple human-significant NTM infections (Johansen *et al.* 2024). Importantly, zebrafish models also recapitulate important host-pathogen interactions and pathophysiological hallmarks of NTM infections (cellular recruitment, granulomatous response, abscess formation) which are also observed in human clinical manifestations of NTM infections (**Figure 1B**). In addition, toxicity assessments in zebrafish provides fast results for *in vivo* toxicity of leading candidates early in the drug discovery pipeline. Moreover, according to the European legislation, zebrafish are not considered as laboratory animals for up to 5 days post-fertilization, which is when uptake and processing of external food start (Ng *et al.* 2005), and are therefore accepted as an ethical replacement for mammalian animal studies in research (<https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32010L0063>). For these reasons, the zebrafish is an excellent representative model for NTM drug discovery (Johansen *et al.* 2024; Bernut *et al.* 2014a; Davis and Ramakrishnan 2009; Habjan *et al.* 2024).

In this review, we will explore recent findings in NTM drug exploration and validation using the zebrafish model platform, and will highlight outstanding research questions that must be addressed by future studies in the fight against NTM infections.

Embryo and adult zebrafish model platforms

In the last decade, the zebrafish model has been increasingly applied to many different infectious disease models of bacterial, viral and fungal pathogens. The zebrafish model platform is significantly more cost-effective than mammalian models, offering significant advantages such

as live-cell imaging in an *in vivo* setting and the high fecundity of zebrafish compared to murine models. As was recently reviewed (Johansen *et al.* 2024), the zebrafish embryo model platform is experimentally tractable from approximately 30 hours post-fertilization and possesses only an innate immune system, primarily consisting of macrophages and neutrophils, for the first few weeks post-fertilization. Importantly, the zebrafish embryo recapitulates important pathophysiological features of NTM infections, including granuloma formation consisting of the accumulation of macrophages and neutrophils to constrain mycobacterial infection (Johansen *et al.* 2024; Davis and Ramakrishnan 2009). It is during these embryonic stages that optical transparency is high, allowing for the real-time *in vivo* imaging of host-pathogen interactions. Comparatively, adult zebrafish have a fully functioning immune system containing both innate and adaptive immunity, including both B and T cells which provides an entire immunological snapshot (Johansen *et al.* 2024). While the optical transparency and real-time *in vivo* imaging capabilities are significantly diminished in an adult zebrafish, due to their larger size the adult zebrafish can be histologically sectioned in longitudinal whole animal cross-sections for both fresh frozen or formalin-fixed paraffin-embedded purposes (Cheng *et al.* 2020). Histological examination of adult zebrafish NTM infection models has shown the formation of highly structured bona-fide granulomas containing a caseated and necrotic core, closely resembling human granuloma structures (Cheng *et al.* 2020; Swaim *et al.* 2006). An extensive summary of the different zebrafish model platform NTM infection models developed to examine disease pathogenesis can be found here (Johansen *et al.* 2024).

Drug treatment methods in the zebrafish model

From a pharmacological perspective, zebrafish are advantageous over mammalian model platforms in that most drugs can be administered through whole-body immersion at the embryonic stage, overcoming the need for more invasive methods (Bernut *et al.* 2014b; Johansen *et al.* 2020a, 2021). The drawback however, is that treatments are required to be completely water-soluble which means it is not amenable for all compounds, and also that the absorption of water-soluble drugs through the epithelium is not representative of other preclinical models or human applications. Microinjection is commonly used in the embryo and can be applied to multiple sites such as the caudal vein, yolk sac, duct of Cuvier and hindbrain ventricle (Johansen *et al.* 2021). Both the caudal vein and duct of Cuvier are commonly used for systemic delivery, with the caudal vein being much more accessible and less invasive for consecutive injections

(Johansen *et al.* 2021). The hindbrain ventricle is an immunologically privileged niche devoid of immune cells and typically used to measure immune cell recruitment. Similarly, the yolk sac is also devoid of tissue-resident immune populations, however the advantage is that administered compounds are dispersed slowly throughout the embryo as the yolk is resorbed during developmental stages.

Application of the adult zebrafish model for drug studies is significantly lower than embryos, likely due to more complex housing arrangements under infectious conditions as well as increased costs associated with rearing embryos through to adulthood. Furthermore, due to the significantly larger size and thick epithelium, optical transparency is significantly diminished meaning that systemic delivery *via* injection is much less feasible. As previously discussed, one of the most common methods for drug treatment in adult zebrafish is whole-body immersion, which is non-invasive and generally well tolerated depending on toxicity. Further, oral administration of adult zebrafish through medicated feed is another minimally invasive alternative and closely mimics human ingestion as a common route of drug administration (Sridevi *et al.* 2014). This provides an advantage over zebrafish embryos, as they do not require feeding during the first week post-fertilization. Adult zebrafish can be intraperitoneally injected with drugs although this is a relatively invasive procedure which requires full anesthesia of adult zebrafish and is difficult to perform across consecutive days. Intramuscular trunk injection is an alternative method in adult zebrafish, however this is more frequently used for vaccination-based studies (Myllymäki *et al.* 2017; Niskanen *et al.* 2020; Oksanen *et al.* 2013). Overall, the applications of embryonic or adult zebrafish model platforms for NTM infection studies are largely based on the drug administration route and biological readouts, as summarized in **Figure 2**. In the following sections, we provide an overview of various therapies tested against NTM species using zebrafish embryo and adult infection models.

Bacterial-directed therapies

Mycobacterium marinum

Anti-tubercular drug activity in zebrafish embryos was originally performed by infecting embryos intravenously with fluorescent *M. marinum*, which were then maintained in the presence of isoniazid (INH), rifampicin (RIF), ethambutol (EMB), moxifloxacin (MOX) or streptomycin (STM) for several days with changing of the drug-supplemented water on a daily basis (Adams *et al.* 2011; Takaki *et al.* 2013). Increased embryo survival was associated with lower bacterial burden

after 4 days of treatment at concentrations that were non-toxic, as judged by fluorescence microscopy and confirmed quantitatively by Fluorescent Pixel Count (FPC) and Colony Forming Unit (CFU) determination (Adams *et al.* 2011). These findings were consistent with human TB data, indicating that clinically relevant anti-TB drugs were efficacious in the zebrafish infection model. This led to the subsequent development of the *M. marinum*-zebrafish embryo model as a platform for *in vivo* anti-tubercular drug discovery (Takaki *et al.* 2012). In the *M. marinum* embryo model, the addition of INH to RIF revealed a further reduction in bacterial burden (Takaki *et al.* 2012), corroborating findings observed for human TB therapy for which synergism is central in shortening treatment length to sterilization. The benzothiazinone lead compounds BTZ043 and PBTZ169, which kill *M. tuberculosis* by targeting the decaprenylphosphoryl- β -D-ribose 2-epimerase DprE1, were associated with a pronounced decrease in the *M. marinum* burden when tested in zebrafish embryos (Makarov *et al.* 2014). While BTZ043-treated embryos showed defects in development, this was not observed after exposure with PBTZ169 at the same concentrations (Makarov *et al.* 2014).

To unravel novel classes of anti-tuberculosis drugs, a chemical library of unique 3D bioinspired molecules was screened to identify mycobacterium-specific Tricyclic SpiroLactams (TriSLa) (Dam *et al.* 2022). Chemical optimization delivered derivatives with nanomolar activity against replicating and non-replicating *M. tuberculosis*. Characterization of isolated TriSLa-resistant mutants, and biochemical studies indicated that TriSLAs are allosteric inhibitors of type II NADH dehydrogenases (Ndh-2) of the electron transport chain, causing an increase in bacterial NADH/NAD⁺ ratios and reduced ATP levels. *In vivo* proof-of-concept studies showed TriSLAs protected zebrafish embryos from lethality upon *M. marinum* infection, emphasizing the vulnerability of Ndh-2 inhibition in mycobacterial infections (Dam *et al.* 2022).

Non-conventional anti-TB molecules have also been tested in the *M. marinum*-zebrafish embryonic model. This is the case for thiostrepton (TSR), an FDA-approved antimicrobial drug, containing a natural thiopeptide which binds to a cleft located between the L11 protein and 23S rRNA of the 50S large ribosomal subunit, perturbing protein synthesis (Wilson 2014). The antibacterial activity of TSR and its derivatives 6'-Fluoro-TSR, 5'-Fluoro-TSR and 12'-Methyl-TSR were investigated in *M. marinum*-zebrafish embryonic platform, where all three compounds strongly reduced bacterial loads and mortality in infected embryos (Zheng *et al.* 2015).

Medium-throughput capacities of the embryo infection model have been exploited to demonstrate the prediction and selectivity of compounds administered orally (Habjan *et al.*

2021). This model was used to screen 240 compounds from an anti-TB hit library for their activity in *M. marinum*-infected zebrafish. Among the most active scaffolds, TBA161 was found to inhibit an aspartyl-tRNA synthetase, blocking protein translation. The target was genetically confirmed in both *M. marinum* and *M. tuberculosis*, with molecular docking proposing possible binding of TBA161 in a pocket adjacent to the enzyme catalytic site (Habjan *et al.* 2021).

These few examples illustrate the advantage of incorporating an early zebrafish model in the TB drug discovery pipeline, which accelerates drug discovery, saving costs and efforts (Carvalho *et al.* 2011; Takaki *et al.* 2012; Ordas *et al.* 2015). Many other new bioactive compounds to improve TB therapy using the zebrafish model infected with *M. marinum* have been assessed over the last two decades and summarized recently (Antunes *et al.* 2024; Habjan *et al.* 2024).

Mycobacterium abscessus

Given the extreme resistance levels of *M. abscessus* to most commonly used antibiotic classes (Johansen *et al.* 2020b), high-throughput drug discovery platforms such as the *M. abscessus*-zebrafish embryo model are highly desirable. Based on previous work with *M. marinum*, experimental conditions for spatiotemporal *in vivo* imaging of *M. abscessus*-infected embryos and their use to assess the efficacy of drug treatments have been developed (**Table 1**). In zebrafish embryos, experiments are usually conducted using the rough morphotype of *M. abscessus*, which induces an acute and lethal infection, as compared to the smooth variant, which is chronic and often non-lethal (Bernut *et al.* 2014a). Originally developed to test the suitability and sensitivity of two clinically relevant drugs, CLR (macrolide) and imipenem (IMP; β -lactam), *M. abscessus*-infected embryos were used to visualize and quantify decreases in cord and abscess formation in the presence of these compounds in a dose- and time-dependent manner (Bernut *et al.* 2014b). This proof-of-concept study demonstrated that zebrafish embryos are suitable for drug efficacy testing against *M. abscessus* (Bernut *et al.* 2014b). Next, the increased efficacy of a drug combination consisting of a β -lactam (Amoxicillin; AMOX or IMP) and a specific β -lactamase inhibitor (avibactam) in zebrafish, further demonstrated the potential of zebrafish embryos as an amenable and relevant preclinical animal model (Dubée *et al.* 2015; Lefebvre *et al.* 2017).

ATP synthase inhibitors

Repurposing anti-tubercular drugs represents an attractive alternative to be explored for future chemotherapeutic developments against *M. abscessus* infection (Degiacomi *et al.* 2019).

In this context, bedaquiline (BDQ), an ATP synthase inhibitor, approved for the treatment of multidrug-resistant TB, was shown to be highly active in zebrafish, despite being bacteriostatic *in vitro* against *M. abscessus* (Dupont *et al.* 2017). Noticeably, a very short period of treatment was sufficient to protect the infected embryos from killing, corroborated with diminished numbers of abscesses and cords, often characterized as pathophysiological traits typifying *M. abscessus* infection in zebrafish (Bernut *et al.* 2014a) (**Figure 3**). Recently, BDQ derivative TBAJ-5307 was discovered as a broad spectrum anti-NTM inhibitor targeting the ATP synthase engine for both fast- and slow-growing NTM laboratory and clinical isolates by depleting intrabacterial ATP (Ragunathan *et al.* 2024). Moreover, this inhibitor was particularly active against *M. abscessus in vitro*, in macrophages and in infected zebrafish, without inducing toxicity. Combining TBAJ-5307 with anti-NTM antibiotics or the oral tebipenem-avibactam pair showed attractive potentiation. The TBAJ-5307-tebipenem-avibactam cocktail was able to effectively kill *M. abscessus*, offering a novel oral drug combination for the treatment of NTM lung diseases (Ragunathan *et al.* 2024).

Transcription inhibitors

In contrast to *M. tuberculosis*, *M. abscessus* is highly resistant to rifampin, an inhibitor of the RNA polymerase. However, screening of libraries of approved pharmaceuticals led also to the selection of rifabutin (RFB), a rifamycin-derived antibiotic, demonstrating activity against *M. abscessus in vitro* (Aziz *et al.* 2017; Pryjma *et al.* 2018). Application of RFB treatment to *M. abscessus*-infected zebrafish embryos demonstrated reduced bacterial burden and abscess formation (Johansen *et al.* 2020a). This supports the practicality of zebrafish as a preclinical model to evaluate the bactericidal efficacy of RFB against *M. abscessus* infection in real-time within the context of innate immunity and strengthens the view that RFB should be considered a repurposing drug candidate for the treatment of *M. abscessus* infections (Ganapathy *et al.* 2019). These results prompted other rifamycin analogues to be tested against *M. abscessus*, particularly rifamycin-O, which showed comparable activity to RFB in zebrafish embryos (Hanh *et al.* 2020b).

Protein synthesis inhibitors

Epetraborole (EPT) is an advanced non-halogenated 3-amino-methyl benzoxaborole and acts as an inhibitor against the essential protein leucyl-tRNA synthetase (LeuRS) in *M. abscessus* (Sullivan *et al.* 2021), blocking protein synthesis. EPT protected zebrafish from lethal *M. abscessus*

infection throughout the 5-day treatment course and was correlated with decreased bacterial burdens beginning from 4 days post-infection. *In vitro*, the combination of EPT with the non-proteinogenic amino acid norvaline limited the rate of EPT resistance in both *M. abscessus* and *M. tuberculosis* (Sullivan *et al.* 2021). The demonstration of norvaline adjunct therapy with EPT for *M. abscessus* infections is promising and could translate to other NTM infections.

Thiostrepton, previously assessed for its activity in *M. marinum*-infected zebrafish (Zheng *et al.* 2015), has also been repurposed for *M. abscessus* infection and was shown to significantly reduce bacterial burden and improve survival in the zebrafish embryo model (Kim *et al.* 2019). The streptogramin Etamycin produced by an *Actinomycetes* species was recently demonstrated to have significant *in vitro* and *in vivo* activity against *M. abscessus*-infected macrophages and particularly zebrafish embryos, with increased survival and reduced bacterial burden (Hanh *et al.* 2020a). Further, there is significant potential for these compounds to be successfully applied to future therapeutic applications against other NTM infections, as judged by their very low minimal inhibitory concentration (MIC) against *M. avium* and *M. intracellulare* (Hosoda *et al.* 2019).

Mycolic acid inhibitors

Mycolic acids represent crucial elements of the mycobacterial cell wall. They are a homologous series of C₆₀₋₉₀ α -alkyl β -hydroxy fatty acids and are primarily found as esters of the non-reducing arabinan terminus of arabinogalactan (Brennan and Nikaido 1995) and as extractable free lipids within the cell wall, mainly associated to trehalose to form trehalose monomycolate (TMM) and trehalose dimycolate (TDM) (Bhatt *et al.* 2007). A phenotypic screen using a library of compounds with activity against *M. tuberculosis* was applied against *M. abscessus*, which identified a piperidinol-based compound, designated PIPD1 (Dupont *et al.* 2016). This compound exhibited a very low MIC against clinical strains of *M. abscessus in vitro* and high activity in infected macrophages and zebrafish. Treatment with PIPD1 correlated with increased embryo survival and decreased bacterial burden and abscesses (Dupont *et al.* 2016). Genetic and biochemical studies indicated that PIPD1 acts by inhibiting the TMM transporter MmpL3. This instigated further research towards the identification of additional MmpL3 inhibitors, such as indole-2 carboxamides (Kozikowski *et al.* 2017) or benzimidazole derivatives. Among the latter, 2-(2-cyclohexylethyl)-5,6-dimethyl-1H-benzo[d]-imidazole, designated EJMCh-6, significantly increased survival of *M. abscessus*-infected zebrafish embryos at low concentrations, further displaying significant reductions in bacterial burden (Raynaud *et al.*

2020). Recently, an unmarked *mmpL3* conditional knock-down mutant was engineered in both smooth and rough *M. abscessus* (Boudehen *et al.* 2023). Using a TetOff-based system, where the addition of anhydrotetracycline (ATc) was associated with a dose-dependent decrease in the production of the MmpL3 protein, intravenous injection of ATc in zebrafish embryos infected with the rough *mmpL3* conditional mutant impeded bacterial growth and correlated with a significant increase in embryo survival (Boudehen *et al.* 2023). This confirms that *mmpL3* is essential for *M. abscessus* growth *in vitro* and *in vivo*. Collectively, these findings highlight that MmpL3 inhibitors hold promise for the development of novel therapeutic agents against *M. abscessus* infections and validate the mycolic acid pathway as an attractive drug target to be further exploited (Alcaraz *et al.* 2023).

Aldehyde dehydrogenase

Disulfiram is a drug that has been utilized as an oral alcohol deterrent in humans, which inhibits the aldehyde dehydrogenase, with potent activity against drug-susceptible and drug-resistant *M. abscessus* strains shown *in vitro* and in macrophages and subsequently confirmed in zebrafish embryos (Winters *et al.* 2022). Treatment was correlated with decreased bacterial loads and enhanced embryo survival (Winters *et al.* 2022). However, the target of disulfiram in *M. abscessus* remains to be confirmed. Overall, these findings suggest that disulfiram may have significant clinical implications for *M. abscessus* management in patients.

Mycobacterium fortuitum

Recent work focusing on the 4-hydroxy-2-pyridone NITD-916 provided proof-of concept that direct inhibitors of the enoyl ACP reductase InhA (an essential enzyme of the mycolic acid biosynthetic pathway), are highly active against *M. abscessus in vitro*, in macrophages and in CF patient-derived airway organoids (Alcaraz *et al.* 2022). NITD-916 was also very effective *in vitro* against *Mycobacterium fortuitum* (Roquet-Banères *et al.* 2023), a rapidly-growing NTM, sometimes encountered in CF patients (Richards and Olivier 2019). This opportunistic pathogen is highly resistant to a large variety of antibiotics, including β -lactams, aminoglycosides, macrolides, tetracyclines, or anti-TB drugs (Shen *et al.* 2018). Strikingly, in zebrafish embryos, a 1-day treatment with NITD-916 significantly reduced the bacterial loads, pathophysiological features such as cords and abscesses, which are associated with worsened disease severity, and led to strong protection of zebrafish infected with *M. fortuitum* (Roquet-Banères *et al.* 2023). In

M. fortuitum, NITD-916 drastically inhibited *de novo* synthesis of mycolic acids in an InhA-dependent manner. Supporting these data, the resolution of the crystal structure of the InhA_{MFO}-NAD-NITD-916 ternary complex confirmed the direct binding of NITD-916 with InhA_{MFO} (Roquet-Banères *et al.* 2023). Overall, these findings in *M. abscessus* and *M. fortuitum* indicate that the mycolic acid pathway (biosynthesis and transport) is a promising drug target for future NTM drug discovery.

Phage-directed therapies

New therapeutic alternatives are needed for the treatment and eradication of multi-drug resistant NTM infections such as *M. abscessus*, particularly in chronically infected patients who fail standard chemotherapy regimens. Phage therapy represents an alternative which includes the administration of therapeutic phages that infect and lyse specific bacteria to eradicate or treat infectious diseases. It offers important advantages over broad-spectrum antibiotics as they are unlikely to adversely affect the host or host commensal microbiota due to their very high specificity towards their bacterial hosts. This subsequently prevents the emergence of antibiotic resistance (Luong *et al.* 2020). The successful application of genetically engineered mycobacteriophages has recently been demonstrated in a CF patient chronically infected with multi-drug resistant *M. abscessus* subspecies *massiliense* (named strain GD01) following bilateral lung transplantation. Intravenous phage treatment was well tolerated and associated with global clinical improvements, reducing the size and abundance of infection foci (Dedrick *et al.* 2019). These findings emphasized the need to develop rapid and reliable pre-clinical models to assess the efficacy of phage therapy against *M. abscessus* clinical strains and to predict successful phage inclusion in a patient prior to clinical application. In this context, a zebrafish-CF model has been assessed as a preclinical animal model to report the complex interplay between phages and bacteria in a CF background in real-time (Johansen *et al.* 2021). Phage Muddy (part of the phage cocktail administered to the CF patient) was found to reduce the pathological signs (abscesses and cords) and increase the survival of zebrafish-CF embryos infected with GD01. Importantly, synergistic activity was obtained when combining phage Muddy with RFB treatment, indicating that phage/antibiotic combination was highly efficacious *in vivo*. This study also reported the essential contribution of innate immunity in determining the success of phage therapy in zebrafish (Johansen *et al.* 2021). Overall, the zebrafish-CF model represents a valuable high-

throughput platform to assess the most successful phage/antibiotic combinations and predictions prior to testing in *M. abscessus* patients.

Host-directed therapies

Antibiotics traditionally act directly on bacteria by killing or inhibiting growth. However, targeting host factors required for the pathogen to survive and replicate represents an alternative to controlling infection without triggering the emergence of drug-resistant strains (Kaufmann *et al.* 2018). Host-directed therapies (HDT) modulate specific pathways or mechanisms such as activation of the host immune system to more efficiently combat infection, or by reducing the symptoms caused by excessive inflammation (Kaufmann *et al.* 2018). There have been many studies into HDT for TB to reach better clinical outcomes through modulation of the antimicrobial host defense mechanisms and by controlling deleterious inflammation (Mahon and Hafner 2015; Machelart *et al.* 2017). In contrast, there has been an increasing number of studies reporting investigation of HDT against *M. marinum* and only very few against other NTM species, presumably due to our limited understanding of the host responses to these NTM infections.

HDT in the *M. marinum*/zebrafish model

Candidate genes identified in the *M. marinum*-zebrafish platform as determinants of mycobacterial disease progression have also been associated with TB severity in humans (Tobin *et al.* 2012; Thuong *et al.* 2017), prompting investigators to exploit the *M. marinum*-zebrafish embryo pairing to perform a chemical screen for active HDT during mycobacterial infection. Using this model, the screening of a library of FDA-approved drugs led to multiple hit compounds able to modulate host immunity to restrict mycobacterial disease, of which clemastine was found to improve mycobacterial control by potentiating the purinergic receptor P2RX7, leading to inflammasome activation and reduced bacterial loads (Matty *et al.* 2019).

An additional screening of FDA-approved drugs revealed that the anticonvulsant carbamazepine stimulates autophagic killing of intracellular mycobacteria within macrophages (Schiebler *et al.* 2015). In zebrafish, treatment with carbamazepine significantly increased the accumulation of autophagosomes and enhanced clearance of *M. marinum* from infected zebrafish embryos (Schiebler *et al.* 2015). Similarly, carbamazepine-induced autophagic killing was observed in mice infected with multi-drug resistant *M. tuberculosis*, indicating that

autophagic enhancement by repurposed drugs provides a viable therapy for the treatment of mycobacterial infections.

Vascular dysfunction encompassing infection-induced angiogenesis, vascular permeability, and hemostasis has been observed in numerous mycobacterial infection systems including the *M. marinum*-zebrafish system (Oehlers *et al.* 2015; Hortle *et al.* 2019). The recruitment of ectopic vasculature to *M. marinum* granulomas can be prevented by treatment with VEGFR inhibitors which reduce bacterial burden (Oehlers *et al.* 2015). Similarly, the treatment of infection-induced vascular leakage reduces bacterial burden and increases correlates of protective immunity (Oehlers *et al.* 2017; Kam *et al.* 2022a). Thrombocyte activation has been proposed to promote mycobacterial growth *in vivo*, and it has been shown that *M. marinum* infection induces thrombocytosis in zebrafish embryos (Hortle *et al.* 2019). In this model, platelet-inhibiting drugs such as aspirin, tirofiban and eptifibatide, reduce the bacterial burden through host-mediated activity, independently of effects on coagulation (Hortle *et al.* 2019). The last set of findings revealed an unanticipated mechanism by which aspirin, a well-tolerated and low-cost drug, may benefit TB and NTM patients.

In addition to inhibiting protein synthesis, thiostrepton (TSR) is also responsible for inducing autophagy-associated LC3-II production in zebrafish, a mechanism that contributes to the elimination of intracellular *M. marinum* (Zheng *et al.* 2015). These findings highlight the dual function of TSR molecules, acting directly by inhibiting protein synthesis in *M. marinum* and indirectly by inducing autophagy. Recently, the anti-mycobacterial effect of the anti-arrhythmic agent amiodarone was validated in *M. marinum*-infected zebrafish (Kilinç *et al.* 2024). Amiodarone induces the formation of autophagosomes and autophagy and reduces the bacterial burden, while failing to directly inhibit mycobacterial growth, thereby confirming the HDT activity of amiodarone *in vivo*.

Mycobacteria are remarkable pathogens able to manipulate host intracellular signaling pathways to escape the host defense. Therefore, pharmacological reprogramming of the immune system represents a potentially powerful approach to fight against mycobacterial infections. Acetylation of histone proteins represents a major mechanism to control DNA accessibility and gene expression (Verdone *et al.* 2005). While histone acetyltransferases (HATs) acetylate lysine residues in histones resulting in a more relaxed chromatin structure associated with increased transcription, histone deacetylases (HDACs) compromise the activity of HATs by removing acetyl groups from highly conserved lysine residues associated with transcriptional

repression. Specific class IIa HDAC inhibitors and pan-HDAC inhibitor Trichostatin has been evaluated in *M. tuberculosis*-infected macrophages, resulting in a significant decrease in intracellular *M. tuberculosis* (Moreira *et al.* 2020). These observations were further validated in the *M. marinum*-zebrafish embryo model, establishing the potential of HDAC inhibitors as novel host-derived therapeutics for mycobacterial infections (Moreira *et al.* 2020).

Recently, the anti-mycobacterial effect of the anti-cancer tamoxifen was confirmed in the *M. marinum* zebrafish infection model. These effects appears independent of the estrogen receptor pathway, but instead appears to act by inducing autophagy and delivering mycobacteria into the lysosomes, thus leading to a reduced bacterial burden (Boland *et al.* 2023). This emphasizes the vast potential of tamoxifen as a repurposed drug to fight against mycobacterial diseases through HDT.

HDT in other NTM/zebrafish models

Sirtuins deacetylases belong to the class III HDAC family, characterized by NAD⁺-dependent deacetylase activity (Fiorino *et al.* 2014). Sirtuin 3 (SIRT3) has recently been shown to participate in the host defense in *M. abscessus* infection (Kim *et al.* 2020). SIRT3 deficiency leads to increased bacterial loads, histopathological and mitochondrial damage and inflammation during infection in mice. Interestingly, resveratrol (RSV), an activator of SIRT1 and SIRT3, significantly inhibits bacterial loads in the lungs of mice as well as in zebrafish embryos (Kim *et al.* 2020). Embryos treated with RSV displayed increased survival, indicating that RSV is an effective compound for treating *M. abscessus* infection. SIRT3 plays a protective role against *M. abscessus* and that SIRT3-targeted activation by RSV promotes antimicrobial defenses by reducing the excessive inflammation and ameliorating mitochondrial oxidative stress during infection (Kim *et al.* 2020).

The adult zebrafish platform for NTM drug discovery and development

Mycobacterium marinum

The *M. marinum* model is the most well-characterized adult zebrafish platform for drug testing and efficacy (Swaim *et al.* 2006). However, few studies have shown the applicability of this platform for the identification and development of drug therapies for NTM infection. One such study has explored the efficacy of common anti-mycobacterial drugs (INH, RIF, EMB, MOX, AMOX) *via* oral ingestion administered for 1 week from 1-week post-challenge with a lethal dose of *M. marinum*. This study showed high bacterial clearance for all drugs tested (except AMOX),

with MOX and INH + RIF (combination) performing best overall, recapitulating human clinical applicability and highlighting the translational applicability of this preclinical model (Sridevi *et al.* 2014). Another study examined the efficacy of EMB on latent *M. marinum* infection. Following oral dexamethasone treatment, the authors were able to induce granuloma breakdown in latent infection, resulting in a depletion of CD4⁺ cells encasing the granuloma and reversion to active *M. marinum* infection. Further, the authors showed that EMB was only active against active *M. marinum* infection whilst INH, AMK and Metronidazole (MET) were ineffective. EMB was ineffective against latent *M. marinum* infection, consistent with our understanding of the role of EMB in preventing mycobacterial replication (Myllymäki *et al.* 2018).

Recent advances in *ex vivo* tissue explants have been explored within the context of the adult zebrafish granuloma, which provides an immunological snapshot of the granuloma micro-environment in real-time (Cronan *et al.* 2018). Using these techniques, the authors were able to culture and longitudinally image *M. marinum*-host immune interactions up to 7 days post-explant, observing bacterial replication and monitoring macrophage epithelization in real-time. Importantly, *in vivo* efficacy of anti-mycobacterial agents do not always reflect the *in vitro* MIC values, partly because the granuloma can act as a barrier preventing drug penetration inside the bacilli (Dartois 2014). Thus, the *M. marinum* granuloma explant model was used to assess the efficacy of anti-mycobacterial drugs, with EMB, RIF and BDQ being highly effective at near MIC values, while other drugs required doses much higher than the MIC values (Cronan *et al.* 2018).

Following a chemical library screen of >1200 compounds in an *M. marinum* zebrafish embryo model where the authors identified clemastine as a potent HDT targeting macrophage transport *via* the P2RX7 receptor, they then applied this to adult zebrafish *ex vivo* granuloma explants (Matty *et al.* 2019). Clemastine reduced the bacterial burden within explanted granulomas in a P2RX7-dependent manner, and combination therapy paired with MOX significantly improved bacterial clearance demonstrating the synergistic role of anti-mycobacterial therapies and HDT in targeting NTM infections.

Mycobacterium abscessus

The adult zebrafish model was applied to understand the host pathophysiology of *M. abscessus* infection (Kam *et al.* 2022b) and subsequently used to explore the role of host vasculature as a pathogenic mechanism (Kam *et al.* 2022a). The authors showed that the hijacking of host angiogenesis to recruit blood vessel growth towards the *M. abscessus* granuloma was present

for the rough but not the smooth variant. Further, the authors applied HDT targeting angiogenesis by using pazopanib (a VEGFR inhibitor), which reduced the distance between the granuloma and nearest blood vessel while also reducing *M. abscessus* burden in the rough morphotype. They also examined the efficacy of AKB-9785 (a VE-PTP inhibitor) to reduce vascular leakage, which similarly to pazopanib was able to reduce the *M. abscessus* burden. However, anti-platelet drugs (aspirin and tirofiban) on *M. abscessus* infection failed to show a reduced bacterial burden. Overall, this study shows that the *M. abscessus* adult zebrafish model can be successfully exploited to discover active HDT and could be applied to antimicrobial studies.

Mycobacterium chelonae

There are only a few studies which have described *M. chelonae* infection in adult zebrafish, most of which were naturally acquired infections identified through routine diagnostic testing (Whipps *et al.* 2014, 2012). To date, one study has explored the efficacy of antimicrobials against *M. chelonae* in an adult zebrafish model (Chang *et al.* 2017). Following intraperitoneal injection of both adult male and female zebrafish with *M. chelonae*, groups were given medicated feed containing either CLR or Tigecycline (TIG) for 14 or 30 days post-infection (short-term and long-term treatment, respectively) and compared to those that were untreated. Both short and long-term treatments with either CLR or TIG reduce the prevalence of granulomas and the presence of acid-fast bacilli. Interestingly, when groups were stratified based on sex, males were much more responsive to both CLR (long-term) and TIG (short-term) with the complete absence of granulomas/acid-fast bacilli, suggesting there are sex-biased responses to NTM drug susceptibility, however these findings warrant further investigation.

Advancements in zebrafish screening for NTM drug discovery

The zebrafish model provides a scalable, vertebrate-based platform for high-throughput screening of anti-NTM compounds, with automation enhancing both precision and throughput. While immersion methods allow non-invasive bacterial introduction (Dalton *et al.* 2017), they suffer from variable dosing, making microinjection a more consistent alternative (Habjan *et al.* 2024). A high-throughput screening model for *M. marinum* and *M. tuberculosis* infection in zebrafish embryos leverages robotic microinjection into the yolk, facilitating batch processing of thousands of eggs (Carvalho *et al.* 2011; Ordas *et al.* 2015; Veneman *et al.* 2013, 2014). Ordas

et al. screened 15 preclinical compounds, identifying four that significantly reduced bacterial burden, with two subsequently validated in murine models, underscoring the translational relevance of zebrafish embryos in TB compound discovery (Ordas *et al.* 2015). Using the same robotic injection system, Habjan *et al.* (2021) introduced *M. marinum* into zebrafish yolk sacs and screened a library of 240 anti-TB compounds. This study identified 14 compounds with strong *in vivo* activity, underscoring the potential of automated screening in uncovering lead compounds. For fast-growing NTM species like *M. abscessus*, yolk injections are unsuitable due to rapid nutrient depletion in the yolk, leading to high embryo mortality within 12 hours post-injection (Habjan *et al.* 2024). Consequently, the bloodstream injection through the caudal vein is a widely used method for the drug test for *M. abscessus* (Hanh *et al.* 2020a, 2020b; Bernut *et al.* 2014b). However, unlike the high-throughput capabilities of yolk injection, bloodstream injection has been challenging to automate, with most studies only testing one compound. Recently, Life Science Methods introduced a robotic system specifically designed for zebrafish embryo injections (Ding *et al.* 2024). This advanced system allows automated injections into multiple sites, including vascular locations like the duct of Cuvier, with efficiency similar to skilled human operators but at twice the speed and with significantly reduced training requirements (Ding *et al.* 2024). Applying this automated injection technology to *M. abscessus* screening could greatly expand the number of compounds tested, accelerating the identification of potential therapeutics for this challenging pathogen (**Figure 4**).

After bacterial infection and compound treatment, an accurate assessment of bacterial burden in infected zebrafish is a crucial step. Bacterial load is typically quantified *via* fluorescence microscopy, with customized macros often used to streamline image acquisition and analysis (Habjan *et al.* 2021; Takaki *et al.* 2012; Knudsen Dal *et al.* 2022; Dupont *et al.* 2017). Fluorescence intensity, indicating bacterial burden for pathogens like *M. marinum* and *M. tuberculosis*, can be rapidly assessed with the COPAS flow cytometer (Veneman *et al.* 2013; Spaink *et al.* 2013; Ordas *et al.* 2015). However, this approach captures only fluorescence intensity, rather than detailed images of the infected embryos. For high-throughput screening, automated image acquisition is essential to maintain both throughput and data quality. Several automated fluorescence microscopy systems are available for HTS applications (Rosa *et al.* 2022). These systems encompass high-content imaging platforms, the VAST Bioimager, and the automated imaging robot. Each offers distinct advantages, with the automated imaging robot exemplifying the potential for rapid, high-throughput image capture with minimal human

intervention. For subsequent image analysis, fluorescent pixels representing bacterial cells can be quantified using software tools such as ImageJ (Adams *et al.* 2011; Knudsen Dal *et al.* 2022), CellProfiler (Habjan *et al.* 2021) or GIMP 2.6 software (Dupont *et al.* 2017).

The automated zebrafish screening platform is designed to streamline the drug discovery process against NTM through the automation of key steps like microinjection and imaging (**Figure 4**). These advancements offer a powerful tool to explore a broader range of drug candidates and dosing regimens with reduced variability and time, enhancing reproducibility and scalability across studies. Altogether, automated zebrafish platforms exemplify the potential of leveraging innovative screening technologies to accelerate the discovery of effective treatments against NTM species, addressing an urgent need in infectious disease research.

Conclusions and future perspectives

The embryonic and adult zebrafish model platforms have enabled the study of pathogenesis and *in vivo* drug discovery for the application to multiple NTM infections. As the emergence of multi-drug resistant NTM infections increases, the need to develop new effective treatments is urgent. Herein, we have presented an overview of the advantages of the zebrafish model which, in recent years, has facilitated important basic research discoveries that have been used as platforms to accelerate the identification of new drug candidates. Moreover, the genetic amenability and wide array of fluorescent reporter zebrafish that can be imaged to model host-pathogen interactions in real-time represent a significant asset over other preclinical animal models. Thus, while the zebrafish embryo model does not replace the gold standard murine models, it complements the space between the *in vitro* cell-based evaluations and more complex *in vivo* models, prior to further examination in mammals. However, whereas most studies have focused on the activity of drugs/treatments on reference strains (which are usually easier to manipulate), future work should also address the activity of compounds on multi-drug resistant clinical NTM isolates circulating within the human population, which represent an emerging global healthcare concern. Additional studies should also dedicate resources into the establishment of zebrafish models of less common opportunistic and pathogenic NTM species to further our understanding of host-pathogen interactions and the identification of effective therapies due to the increasing prevalence of NTM infections globally. However, other important questions remain to be

addressed. For instance, how can we improve the clinical translation of *in vivo* drug therapies in zebrafish to human applications? How can we utilize emerging technologies and artificial intelligence to increase the high-throughput capacity of drug discovery for NTM infections in the zebrafish model platforms? Can MALDI-imaging be developed to determine the concentration and bioavailability of drugs (and their metabolites) in drug-treated zebrafish embryos? Since persistent mycobacteria are often associated with treatment failure and disease relapse due to phenotypic drug tolerance, how can we improve the zebrafish model for screening purposes to target mycobacterial persisters?

ACKNOWLEDGMENTS

We apologize to authors whose work could not be cited owing to space limitations. This study was supported by the Fondation pour la Recherche Médicale (Equipe FRM EQU202103012588) to LK. MDJ was supported by a Chancellor's Research Fellowship (CRF) provided by the University of Technology Sydney. Figures were made using BioRender.

DECLARATION OF INTEREST

MDJ, CH and LK have no conflict of interest to declare. YD works for Life Science Methods that develops and sells microinjection, sorting and imaging robots for zebrafish applications.

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FIGURE LEGENDS

Figure 1. The use of zebrafish models to study pathogenesis of NTM and evaluate the therapeutic efficacy of drugs *in vivo*. (A) Classification of major NTM based on their growth rate. The species previously investigated in the embryo or adult zebrafish models are indicated using specific zebrafish icons next to their name. Figure adapted from Johansen *et al.* 2024. (B) A comparison of the different zebrafish models and their ability to recapitulate major symptoms underpinning NTM human diseases.

Figure 2. Comparison of the embryo and adult fish models to evaluate the *in vivo* efficacy of test compounds. The major drug administration routes and the biological readouts to measure the efficacy of compounds are indicated in the left and right panels, respectively. ✓, adapted model, ✓✓, widely used model, X, not suitable model.

Figure 3. Experimental design to evaluate the antimycobacterial effect of drug treatment in NTM-infected embryos. Examples are provided here with *M. abscessus* and *M. marinum*. Prior to infection, the test compounds are assessed for toxicity by determining a disease score in embryos (1). The embryos are then microinjected with fluorescent mycobacteria (2) and exposed to the test compounds (3). The activity of the compounds is then evaluated by determining the effect on embryo killing (survival curves and whole embryo imaging) (4a), as illustrated with the rough variant of *M. abscessus* treated with bedaquiline (BDQ) (Adapted from Dupont *et al.* 2017). The duration of the treatment is indicated with a colored line along the x axis (1 day in pink and 3 days in green). Embryo survival was monitored daily over an 11-days period. Each treatment group was compared against the untreated control group with significant differences calculated using the log rank (Mantel-Cox) statistical test for survival curves ***, $P \leq 0.001$. The activity of the compounds can also be assessed by measuring the bacterial burden through CFU or FPC determination, as exemplified here with *M. marinum* treated Tricyclic SpiroLactams (4b) and by monitoring the evolution of pathological symptoms, including cord formation, abscess formation and granulomas, as illustrated in *M. abscessus*-infected embryos (4b).

Figure 4. An automated zebrafish screening platform for drug discovery against NTM. The workflow begins with the collection of zebrafish eggs at 0 day post-fertilization (dpf). NTM

pathogens, such as *M. abscessus*, are cultured, fluorescently labeled, and prepared for infection. Automated microinjection of *M. abscessus* into the bloodstream of zebrafish embryos enables precise infection modeling. At 1 day post-infection (dpi), test compounds are administered by adding them to the water for systemic exposure or by injecting them directly into individual embryos. Finally, an automated imaging system captures high-resolution images to quantify bacterial load, offering an efficient and reproducible readout for evaluating drug efficacy.

Table 1. *In vivo* assessment of drug efficacy against *M. abscessus* and *M. fortuitum* in zebrafish embryos associated with increased zebrafish lifespan and/or decreased bacterial burden. ND, not described.

Drug	Antibiotic/chemical class	Targeted Pathway	NTM tested	Reference
Clarithromycin	Macrolide	Protein synthesis	<i>M. abscessus</i>	(Bernut <i>et al.</i> 2014b)
Clarithromycin + Omadacycline	Macrolide + tetracycline	Protein synthesis	<i>M. abscessus</i>	(Bich Hanh <i>et al.</i> 2021)
Clarithromycin + Rifaximin	Macrolide + Rifamycin	Protein synthesis + Transcription	<i>M. abscessus</i>	(Goh <i>et al.</i> 2023)
Azithromycin	Macrolide	Protein synthesis	<i>M. abscessus</i>	(Nie <i>et al.</i> 2020)
Epetraborole	Benzoxaborole	Protein synthesis	<i>M. abscessus</i>	(Sullivan <i>et al.</i> 2021; Nguyen <i>et al.</i> 2023)
DS86760016	Benzoxaborole	Protein synthesis	<i>M. abscessus</i>	(Nguyen <i>et al.</i> 2023)
Contezolid (MRX-I)	Oxazolidinone	Protein synthesis	<i>M. abscessus</i>	(Gao <i>et al.</i> 2023)
Thiostrepton	Thiopeptide	Protein synthesis	<i>M. abscessus</i>	(Kim <i>et al.</i> 2019)
Etamycin	Streptogramin	Protein synthesis	<i>M. abscessus</i>	(Hanh <i>et al.</i> 2020a)
Imipenem	β -lactam	Peptidoglycan synthesis	<i>M. abscessus</i>	(Bernut <i>et al.</i> 2014b)
Imipenem + Avibactam	β -lactam + β -lactamase inhibitor	Peptidoglycan synthesis	<i>M. abscessus</i>	(Lefebvre <i>et al.</i> 2017)
Amoxicillin	β -lactam	Peptidoglycan synthesis	<i>M. abscessus</i>	(Dubée <i>et al.</i> 2015)
Amoxicillin + Avibactam	β -lactam + β -lactamase inhibitor	Peptidoglycan synthesis	<i>M. abscessus</i>	(Dubée <i>et al.</i> 2015)
Bedaquiline	Diarylquinoline	ATP synthesis	<i>M. abscessus</i>	(Dupont <i>et al.</i> 2017)
TBAJ-5307	Diarylquinoline	ATP synthesis	<i>M. abscessus</i>	(Ragunathan <i>et al.</i> 2024)
Sudapyridine (WX-081)	Diarylpyridine	ATP synthesis	<i>M. abscessus</i>	(Nie <i>et al.</i> 2023)
Rifabutin	Rifamycin	Transcription	<i>M. abscessus</i>	(Johansen <i>et al.</i> 2020a)
Rifamycin O	Rifamycin	Transcription	<i>M. abscessus</i>	(Hanh <i>et al.</i> 2020b)
Disulfiram	Antialcooholic	ND	<i>M. abscessus</i>	(Winters <i>et al.</i> 2022)
PIPD1	Piperidinol analogue	Mycolic acid transport	<i>M. abscessus</i>	(Dupont <i>et al.</i> 2016)
EJMCh-6	Benzimidazole	Mycolic acid transport	<i>M. abscessus</i>	(Raynaud <i>et al.</i> 2020)
NITD-916	4-hydroxy-2-pyridone	Mycolic acid biosynthesis	<i>M. fortuitum</i>	(Roquet-Banères <i>et al.</i> 2023)

A

**Slow-growing mycobacteria
(>7 days)**

Pathogens

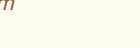
- *M. tuberculosis* complex *
- *M. leprae* *
- *M. ulcerans*



* not part of NTM classification

Opportunistic pathogens

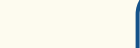
- *M. avium* complex
- *M. gastri*
- *M. haemophilum*
- *M. kansasii*
- *M. marinum*
- *M. scrofulaceum*
- *M. simiae*
- *M. xenopi*



**Rapid-growing mycobacteria
(<7 days)**

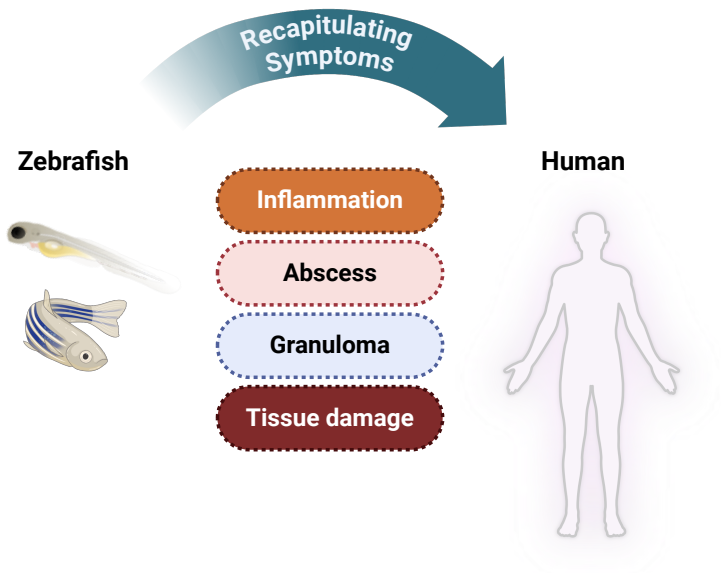
Opportunistic pathogens

- *M. abscessus* complex
- *M. chelonae*
- *M. fortuitum*
- *M. peregrinum*





Saprophytes



- *M. phlei*
- *M. smegmatis*
- *M. vaccae*

B

Drug administration route

			
Waterborne exposure		✓✓	✓
Food		✗	✓✓
Microinjection	Caudal vein	✓✓	✗
	Duct of Cuvier	✓✓	✗
	Hindbrain ventricle	✓	✗
	Yolk sac	✓	✗
	Muscle	✓	✓
	Peritoneum	✗	✓

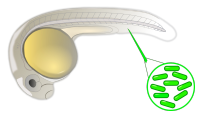
Biological readout

			
Survival		✓	✓
Real time Imaging		✓✓	✗
RNA		✓✓	✓
Bacterial load (Colony Forming Unit)		✓	✓
Bacterial load (Fluorescent quantification by Pixel Count)		✓✓	✗
Histology		✓	✓✓
Flow cytometry		✓	✓✓

1 **Toxicity assesment**

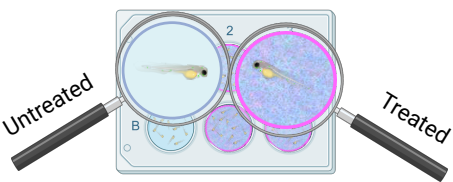
- Disease Score Toxicity Criteria:
- Impaired response to stimuli
 - Abnormal development
 - Abnormal physiological function
 - Yolk opacity
 - Edema

2 **Microinjection of fluorescent NTM in 30 hpf embryo**



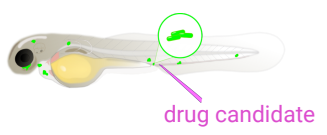
3 **Drug candidate treatment**

Waterborne exposure

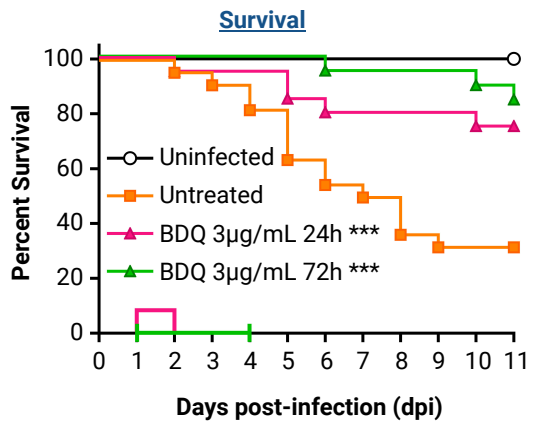


or

Microinjection



4A **In vivo efficacy of bedaquiline (BDQ) against *Mycobacterium abscessus* embryos**

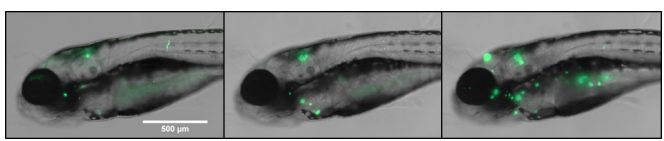


Real-time infection monitoring

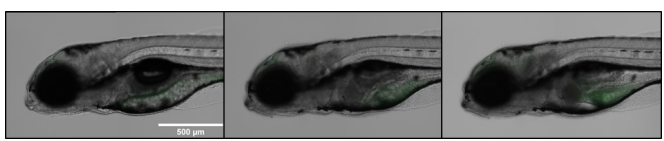


M. abscessus (Rough morphotype)

Untreated



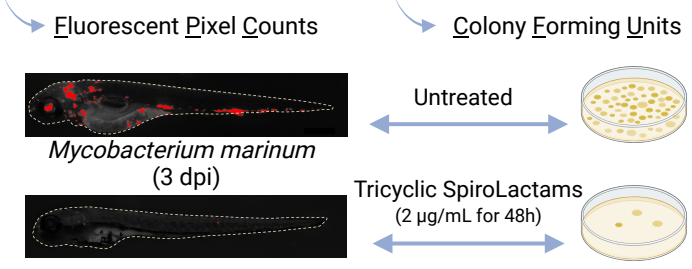
BDQ (3 µg/mL for 72h)



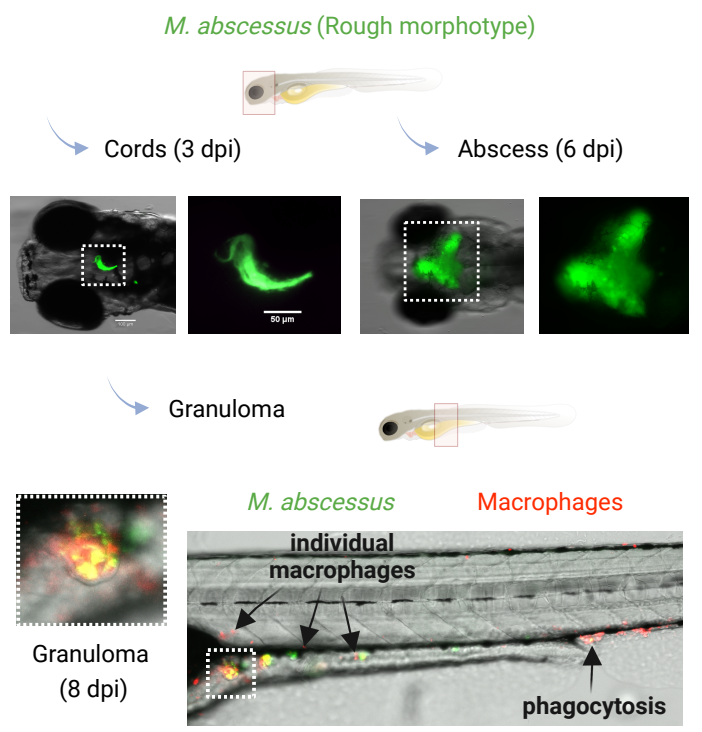
3 dpi 4 dpi 6 dpi

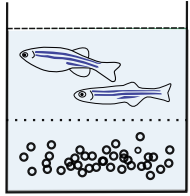
4B **Impact of drug treatments on the evolution of NTM infection**

Bacterial load



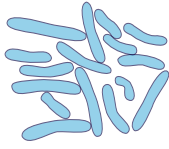
Symptom observation





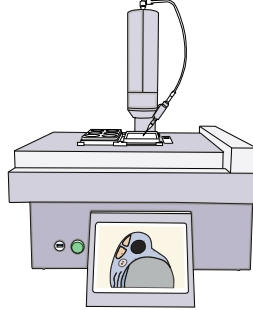
Egg collection

0 dpf



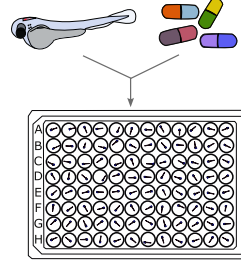
NTM preparation

0-2 dpf



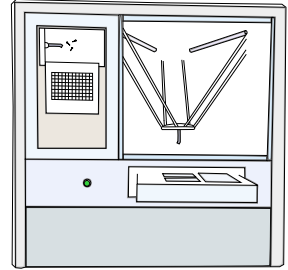
Automated injection

1-2 dpf



Compound treatment

1 dpi



Automated imaging

5 dpf