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# Embryonic and larval zebrafish models for the discovery of new bioactive compounds against tuberculosis

**Stella S. Antunes [1], Gabriel Forn-Cuní [2], Nelilma C. Romeiro [1], Herman P. Spaink [2], Fons J. Verbeek [3], Michelle F. Muzitano [1,*]**

[1] Institute of Pharmaceutical Sciences, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
[2] Institute of Biology Leiden, Leiden University, Leiden, the Netherlands
[3] Leiden Institute of Advanced Computer Science, Leiden University, Leiden, the Netherlands

**Tuberculosis (TB) is a world health challenge the treatment of which is impacted by the rise of drug-resistant strains. Thus, there is an urgent need for new antitubercular compounds and novel approaches to improve current TB therapy. The zebrafish animal model has become increasingly relevant as an experimental system. It has proven particularly useful during early development for aiding TB drug discovery, supporting both the discovery of new insights into mycobacterial pathogenesis and the evaluation of therapeutical toxicity and efficacy *in vivo*. In this review, we summarize the past two decades of zebrafish–*Mycobacterium marinum* research and discuss its contribution to the field of bioactive antituberculosis therapy development.**

**Keywords: tuberculosis; zebrafish; Mycobacterium marinum; drug discovery**

## Tuberculosis

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*) that resulted in 1.3 million deaths in 2022 alone. Currently, drug-susceptible TB is a treatable disease that requires a combination therapy treatment of rifampicin, isoniazid, ethambutol, and pyrazinamide for 6 months, although the Centre for Disease Control recently approved a shorter, 4-month therapeutic option for pulmonary TB comprising rifapentine, moxiflacin, isoniazid, and pyrazinamide.(p1) These long treatments cause a significant economic burden and are prone to treatment failure and dropout, which, together with the fact that no considerable changes in the first-line drug treatment of TB have been made in decades, have resulted in the rise of (multi)drug resistance: the major threat to TB control efforts. Therefore, there is an urgent need for shorter and more effective therapies for not only drug-sensitive, but also drug-resistant TB.(p2)

In this review, we summarize and discuss notable studies that used zebrafish embryonic and larval stages infected with *M. marinum* to assess bioactive compounds or to improve TB therapy between 2002 and 2023, highlighting the relevance of this model to achieve this goal. First, we focus on toxicity assays using zebrafish larvae (Table 1) and then consider the use of zebrafish infected with *M. marinum* to test antituberculosis drugs, as well how such models have been used in high-throughput settings and to discover novel approaches to TB therapies, such as nanoparticle (NP)-based drug delivery and host-directed therapies (Table 2).

## Toxicity assays for antituberculosis drugs

The detection of side and toxic effects resulting from the use of new bioactive compounds is a key factor in drug discovery.(p3) In this context, zebrafish (*Danio rerio*) larvae are a well-recognized model to study acute, chronic, and developmental toxicity.(p4) However, differences between toxicity protocols exist, which vary in terms of exposure time and the route of administration of the compounds tested. The most common and simple route

* *Corresponding author. Muzitano, M.F. (mfmuzitano@macae.ufrj.br)*


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**TABLE 1**

**Anti-TB compounds evaluated through toxicity tests in zebrafish embryos.**

| Drug candidate(s) | Starting test age | Administration route | Length of exposure | Toxicological parameters analyzed | Notable results | Refs |
|---|---|---|---|---|---|---|
| Nitrothiazolyl carboxamide analogs (compound 7, 14, and 17a); *N*-linked aminopiperidine-based (compounds 17b, 23, and 26); aminopiperidine linker with piperazine derivatives (compound 9 and 11) | 3 dpf | Waterborne exposure | 4 h | Cardiotoxicity (heart rate variation, atrioventricular dissociation, and assessment of arrhythmogenic potential) | Completely devoid of cardiotoxicity up to 30 μM<br>Showed no significant cardiotoxicity until 30 μM<br>Compound 9 showed mild toxicity at 30 μM and compound 11 showed mild toxicity at 10 μM and moderate toxicity at 30 μM | (p10),<br>(p11),<br>(p56) |
| Thiazole derivatives (compound 27, 31, and 36); carboxamide analogs (compound 18, 25, and 27); quinolone-aminopiperidine analogs (compound 45 and 47); acridine derivative (compound 6) | 3 dpf | Waterborne exposure | 4 h | Cardiotoxicity (hERG channel inhibition) | Completely devoid of cardiotoxicity up to 30 μM;<br>Showed no signs of cardiotoxicity;<br>Compound 45 found to be safe<br>Compound 47 had no cardiotoxicity at 10 μM, 3 μM, or 1 μM, only showing significant cardiotoxicity above 30 μM | (p12),<br>(p13),<br>(p57),<br>(p58) |
| Thioridazine in PLGA NPs | 3 dpf | Intravenous injection | 4 days | Mortality<br>Developmental toxicity (swim bladder inflation, yolk discoloration, pericardial edema)<br>Cardiotoxicity (heart rate variation)<br>Cytotoxicity (necrosis) | No toxicity detected | (p14) |
| Series of 2-(quinoline-4-yloxy) acetamides (compounds 5e, 5 m, and 5 s) | 24 hpf | Waterborne exposure | 48 h | Cardiotoxicity (heart rate variation) | No signs of cardiotoxicity at 1 and 5 μM | (p59) |
| Dithiocarbamates (Fc14-594A and Fc14-584B) | 1–2 hpf | Waterborne exposure | 5 days | Mortality<br>Developmental toxicity (hatching rate, pericardial edema)<br>Morphological alterations (yolk sac, general shape, normal movement)<br>Cardiotoxicity (heart rate variation)<br>Tissues histopathology | Fc14-584B well tolerated and safer at higher concentrations compared with Fc14-594A. At 300 μM, Fc14-584B showed mild abnormalities in body shape (curving of back) and heartbeat.<br>Eggs treated with Fc14-594A at 30 μM did not survive beyond 3 dpf | (p6) |
| Carboxamide analog (Labio_16) and Quinoline analog (Labio_17) | 2 hpf | Waterborne exposure | 2 and 5 days | Cardiotoxicity (heart rate variation) | Labio_16 showed no cardiotoxicity<br>Labio_17 showed dose-dependent cardiotoxicity | (p3) |
| Bedaquiline | 2 dpf | Waterborne exposure | 2 days | Cardiotoxicity (ventricular function – heart rate, stroke volume, cardiac output and fractional shortening) | Presence of cucurbit[7]uril alleviated inherent cardiotoxic effects of bedaquiline | (p9) |
| Serie of 3,4-dihydroquinazolin-4-ones (compound 9n, 9p-s, 9u and 9w) | 2 hpf | Waterborne exposure | 2 and 5 days | Cardiotoxicity (heart rate variation)<br>Morphological alterations (body length, ocular distance, and surface area of eyes)<br>Neurotoxicity (locomotor activity) | Compounds 9n, 9q, 9u, and 9w did not change heartbeat rates at 2 dpf at 3 μM<br>None of compounds tested had neurotoxicity signs<br>Except for compound 9 s, none of compounds showed morphological alterations | (p60) |
| 14 compounds of monothiocarbamate and dithiocarbamate series | 24 hpf | Waterborne exposure | 5 days | Mortality<br>Developmental toxicity (hatching rate, pericardial edema, swim bladder development)<br>Morphological alterations (yolk sac, general shape)<br>Cardiotoxicity (heart rate variation)<br>Neurotoxicity (locomotor activity) | Compounds 1 and 5 up to 400 μM and compounds 3, 5, 7, and 10 up to 500 μM exhibited no or minimal toxicity | (p17) |

**TABLE 1 (*CONTINUED*)**

| Drug candidate(s) | Starting test age | Administration route | Length of exposure | Toxicological parameters analyzed | Notable results | Refs |
|---|---|---|---|---|---|---|
| Rifabutin | 2 dpf | Waterborne exposure | 24 h | Morphological alterations | No toxicity detected | (p53) |
| Gold NPs | Eight-cell stage | Waterborne exposure | 3 days | Mortality | Gold NPs at 50 μg/mL showed less toxicity | (p61) |
| Clofazamine | 2 dpf | Waterborne exposure | 2 or 7 days | Histological examination (cardiotoxicity accompanied by abnormal cell growth, cell death, and inflammation)<br>Transcriptome analysis | Co-administration of *N*-acetylcysteine at 100 μM had protective effect against clofazimine-induced cardiac dysfunction | (p18) |
| Aminopyrimidine PknB inhibitors | 2 dpf | Waterborne exposure | 5 days | Mortality<br>Morphological alterations | Eggs treated with 5 μM of inhibitors 6, 9, or 20 showed survival at or near 100% 5 days post treatment | (p7) |
| Tetracyclic compound TBA161-C | 1 dpf | Waterborne exposure | 5 days | Mortality<br>Morphological alterations | No toxic effects up to 100 μM | (p29) |
| Peptide-Angie1; granulysin-derived peptide (Gran1) | 24 hpf | Waterborne exposure | 24 h | Cytotoxicity (lysis and/or necrosis)<br>Developmental toxicity (delay and/or malformations)<br>Cardiotoxicity (heart edema and/or reduced absent circulation)<br>Neurotoxicity (escape movements) | Angie1 in antimicrobial activity concentrations (1, 10, and 100 μM) showed no toxicity<br>Gran1 neither affected embryo viability nor caused sublethal toxicity | (p15), (p16) |
| Polymeric micelle-formulated nitronaphthofuran | 2 dpf | Posterior caudal vein injection | 4 days | Developmental toxicity (general body shape, blood flow, pericardial edema, swim bladder inflation)<br>Neurotoxicity (equilibrium, escape response to light touch)<br>Cardiotoxicity (heart rate variation) | PM-C7, PM-C11 and PM-C12 at maximum concentration tested (50 mg/kg) showed low levels of toxicity, with mortality not exceeding 16% | (p4) |
| Polymeric micellar NPs encapsulating bedaquiline | 2 dpf | Posterior cardinal vein injection | 24 h | Mortality | NPs exhibited 10% better survival in zebrafish compared with free drug | (p38) |
| Furanyl pyrazolo [3,4-b] quinoline-5-one derivatives | 6 hpf | Waterborne exposure | 5 days | Teratogenicity | P2, P4, P5, P6, and P10 at 0.5 μM did not show any abnormalities | (p62) |
| 6 ATP-competitive kinase inhibitors | 28 hpf | Waterborne exposure | 4 days | Mortality (without heartbeat) and drug-induced side effects (absence of response to mechanical stimulation, edema, tail curvature malformations, or cranial malformations) | GSK1379722A, GW560109X, GW576924A, GW635815X compounds were considered nontoxic | (p63) |

**TABLE 2**

**Evaluation of anti-TB drugs approaches using zebrafish infected with *Mycobacterium marinum*.**

| Type of research | Zebrafish stage | Injection site | Drug and concentration tested | Route of drug administration | Notable results | Refs |
|---|---|---|---|---|---|---|
| Drug screening | 50 hpf | Embryos: yolk circulation valley | Coumarin analog CCA34 (15 μM) | Waterborne exposure | Inhibited bacterial proliferation by FadD32 inhibition | (p38) |
| | 28 hpf | Caudal vein infection | Benzothiazinone BTZ043 and benzothiazinone analog PBTZ169 (25 or 50 nM) | Waterborne exposure | Both compounds reduced bacterial burden. BTZ043 showed developmental abnormalities | (p40) |
| | | Yolk sac circulation | Carbamazepine (50 μM) | Waterborne exposure | Stimulated autophagy and decreased *M. marinum* burden | (p64) |
| | Up to 1024-cell stage | Yolk robotic injection | 15 preclinical lead GSK compounds (10 μM) | Waterborne exposure | Some drugs that had positive uptake and efficacy in zebrafish, were also active in an *in vivo* rodent model | (p37) |
| | 27–31 hpf | Caudal vein injection | Dithiocarbamate Fc14-584 (300 μM) | Waterborne exposure | Treatment significantly decreased *M. marinum* burden | (p6) |
| | 24 hpf | Caudal vein injection | Bis-substituted cyclam derivative compound 21b (10 μM) | Waterborne exposure | Treatment reduced bacterial load *in vivo* | (p41) |
| | 2–8-cell stage | Robotic yolk or caudal vein injection | Ethionamide (0.01, 0.1, and 1 μM); isoniazid (1, 10, and 100 μM); TB Alliance library compounds C1, C2, C3, C4, C5, and C6 (2.5, 5, and 10 μM) | Waterborne exposure | Most of TB Alliance compounds tested were prodrugs. Ethionamide and isoniazid were more effective against genetically modified *M.marinum* | (p30) |
| | 2–32-cell stage | Robotic yolk injection | Tetracyclic compound TBA161-C (1, 3, and 10 μM) | Waterborne exposure | TBA161-C caused significant reduction in *M. marinum* burden in dose-dependent manner | (p29) |
| | 28–30 hpf | Caudal vein injection | Oridonin (35 μM) | Waterborne exposure | Treatment reduced *M. marinum* load in zebrafish | (p65) |
| | 30 hpf | Blood island injection | Ampicillin (100 μg/ml), avibactam (225 μg/ml) and combination of ampicillin/ avibactam | Duct of Cuvier | Ampicillin/avibactam combination reduced bacterial load. Drugs alone had no significant effect on infection | (p39) |
| | 2 dpf | Duct of Cuvier | L-tyrosine (10 μM) | Waterborne exposure | Reduced bacterial burden levels | (p66) |
| | 1 hpf | Yolk robotic injection | 1,2,4-oxadiazole derivative compounds: 36.0, 36.1, and 36.3 (10, 20, and 30 μM) | Waterborne exposure | Results suggested that compound 36.0 suppresses *M. marinum* virulence by inhibiting LipY lipase activity | (p67) |
| | 1 hpf | Yolk robotic injection | Benzothiazole compounds BT-08 (1, 3, and 10 μM) and BT-37 (1 μM) | Waterborne exposure | BT-08 showed dose-dependent efficacy in reducing bacterial burden. BT-37 was more potent derivative and inhibited novel essential drug target: protein MMAR_0407 (Rv0164) | (p68) |
| | 72 hpf | Caudal vein injection | Syringaldehyde (0.5 mM) | Waterborne exposure | Restricted proliferation of *M. marinum* in zebrafish | (p69) |
| HTS | 16–512-cell stage | Yolk robotic injection | Combination of rifampicin (200 μM) and isoniazid (2 mM) | Waterborne exposure | Injection of 2000 embryos per hour and drug screening | (p45) |
| | 36–48 hpf | Caudal vein injection | Isoniazid, rifampicin, ethambutol and moxifloxacin (all at 400 μM) | Waterborne exposure | Rapid *in vivo* drug screening with novel fluorimetry platform | (p34) |
| | 30 hpf | Hindbrain ventricle injection | Isoniazid | Waterborne exposure | High-throughput evaluation of pathogenesis and antimicrobial efficacy | (p33) |
| | 30–48 hpf | Caudal vein injection | Ethambutol, moxifloxacin, and rifampicin (all at 400 μM) | | | |
| | 16–128-cell stage | Yolk robotic injection | Rifampicin (12, 24, and 200 μM) | Waterborne exposure | Injection of 2500 embryos per hour and automated tracking of infection | (p42) |
| | 2 dpf | Medium with *M. marinum* strain | Rifampicin, pretomanid, delamanid, and experimental anti-mycobacterial drugs (all at 10 μM) | Waterborne exposure | Naturally infected zebrafish can be used for rapid anti-TB drug screening | (p44) |

**TABLE 2 (*CONTINUED*)**

| Type of research | Zebrafish stage | Injection site | Drug and concentration tested | Route of drug administration | Notable results | Refs |
|---|---|---|---|---|---|---|
| NPs as drug delivery | 48–52 hpf | Caudal vein injection | Rifampicin-loaded PLGA NPs (14.4 ng/kg–12 mg/kg) | Caudal vein injection | Rifampicin NPs decreased bacterial burden and enhanced embryo survival | (p47) |
| | | | Rifampicin (200 μg/mL); thioridazine-loaded PLGS NPs (32 mg/kg TZ) | Waterborne exposure, caudal vein injection | PLGA-TZ/RIF bath combination treatment improved survival and bacterial burden | (p14) |
| Different NPs as drug delivery vehicles | 2 dpf | Posterior cardinal vein injection | Rifampicin nanoformulations based on MPEO-*b*-PCL (10 mg/kg) | Caudal vein injection | Rifampicin NPs well tolerated and more efficient compared with free rifampicin | (p46) |
| Nanobiotics treatment | 30 hpf | Caudal vein injection | Clofazamine (5 mg/l) and isoniazid (0.1 mg/ml) nanobiotics | Caudal vein injection | Significantly reduced bacterial burden and granuloma number | (p36) |
| Polymersomes loaded with rifampicin for drug delivery | 28–30 hpf | Caudal vein injection | Polymersome-encapsulated rifampicin (3.6 mM) | Duct of Cuvier | Significantly reduced *M. marinum* burden at lower doses compared with free rifampicin | (p50) |
| ALG-AgNPs | 26–28 hpf | Caudal vein injection | ALG-AgNPs (200 μg/ml) | Waterborne exposure | Reduced mycobacterial burden and were nontoxic for zebrafish | (p49) |
| Polymeric micelle-formulated nitronaphthofuran derivatives for drug screening | 48–52 hpf | Caudal vein injection | PM-C7, PM-C11, PM-C12 (50 mg/kg), and PM-RIF (20 mg/kg) | Caudal vein injection | Significantly reduced bacterial burden and improved survival in infected embryos | (p4) |
| Peptomicelle-based formulation as new drug delivery system | 72 hpf | Neural tube | Peptomicelle-encapsulated pretomanid derivative D (37.5 ng) | Posterior cardinal vein injection | Resulted in >50% of fish surviving after 7 dpf | (p48) |
| NPs as drug delivery systems | 2 dpf | Posterior cardinal vein injection | Polymeric micellar NPs encapsulating bedaquiline (1.1 ng for posterior cardinal vein and 2.5 ng for neural tube treatment) | Posterior cardinal vein injection | Bedaquiline NPs showed improved survival and higher reduction in bacterial burden compared with free drug | (p32) |
| | 3 dpf | Neural tube | | Neural tube | | |
| HDT | 2 dpf | Embryos: caudal vein and trunk injection | Pazopanib (250 nM) and SU5416 (250 nM) | Waterborne exposure | Reduced bacterial burden; could be used as adjunctive therapies | (p28) |
| | 36–48 hpf | Venous plexus | Cyclic nitroxide antioxidant MetT (1 mM) | Waterborne exposure | Inhibited growth and dissemination of *M. marinum* | (p35) |
| | 2 dpf | Caudal vein injection | Clemastine fumarate (5 μM) | Waterborne exposure | Reduced intracellular bacterial burden through potentiation of receptor P2RX7 | (p43) |
| | 28 hpf | Caudal vein injection | Rifabutin (5 μM or 10 μM) | Waterborne exposure | AhR inhibition (CH-223191) promoted enhanced rifabutin antimycobacterial activity | (p53) |
| | 43 hpf | Duct of Cuvier injection | Class IIa HDAC inhibitor TMP195 (10 μM); pan-HDAC inhibitor Trichostatin A (30 nM) | Waterborne exposure | Pretreatment with HDAC inhibitors reduced bacterial burden | (p52) |
| Host-targeting drug to prevent necrosis in TB | 2 dpf | Caudal vein injection | Metformin (20 μM) | Waterborne exposure | Inhibited TNF-elicited mROS and macrophage necrosis | (p54) |
| HDT | 30 hpf | Blood island injection | Tamoxifen (2.5, 5, and 10 μM) | Waterborne exposure | Reduced bacterial burden modulating autophagy via lysosomal degradation pathway | (p51) |
| | | Blood island injection | Amiodarone (5, 10, and 20 μM) | Waterborne exposure | Reduced bacterial burden at 5 and 10 μM without toxicity signs | (p70) |
| | 3 dpf | Tail fin injection | Amiodarone 5 μM | | Autophagy-inducing compound | |





GENE-TO-SCREEN (BLUE)

of exposure for drugs in zebrafish studies is waterborne exposure, that is, dissolving the drug directly in the water medium, although intravenous injection can also be used. To standardize the different protocols, initiatives such as the Fish Embryo Acute Toxicity Test (FET) have been developed and accepted as a guideline by the Organisation for Economic Co-operation and Development (OECD), under the Test Guideline 236. The FET test has high sensitivity and is a nice bridge between *in vitro* toxicity tests and toxicity tests in higher mammals.[p5] Table 1 summarizes studies published regarding the evaluation of anti-TB compounds through toxicity tests in zebrafish embryos.

Thus, zebrafish larvae are commonly used to test the toxicity of drugs for which antitubercular effects have been demonstrated *in vitro* in an organism with a high percentage homology to humans. For example, toxicity tests for the dithiocarbamates compounds Fc14-594A and Fc14-584B, inhibitors of β-carbonic anhydrase of *Mtb*, were tested for developmental toxicity and the tissues effect were also evaluated using histopathological studies.[p6] Similarly, given the catalytic domain identity of 75% compared with human kinases, zebrafish was selected as a relevant model for assessing kinase inhibitor toxicity, mortality rates, and morphological alterations.[p7]

Toxicity assays in zebrafish provide relevant and fast results related to the *in vivo* toxicity of compounds early in the drug discovery pipeline. Polypharmacological effects or specific metabolites are difficult to predict without a complete organism, as exemplified by studies in which drug formulations with minimal cytotoxicity using infected bone marrow-derived macrophages (BMDMs) or THP-1 cells showed strong toxicity in zebrafish larvae.[p4],[p7] The advantage of using a whole organism is also beneficial for evaluating specific toxicities, such as cardiotoxicity.[p8] For example, bedaquiline is one of the drugs most recently approved for the treatment of MDR-TB, but the US Food and Drug Administration (FDA) warned of its increased mortality and cardiotoxicity. Zebrafish larvae treated with bedaquiline exhibited cardiac dysfunctions, such as reducing stroke volume, heart rate, cardiac output and fractional shortening at high concentrations.[p9] At the genetic level, cardiotoxicity was predicted by analyzing the drug effect on the function of the human ether-a-go-go-related gene (*hERG*), a zebrafish ortholog to the human gene.[p10],[p11] Therefore, drug-induced potential pro-arrhythmic, hERG channel inhibition, and QTc prolongation were evaluated in this model and successfully translated to human effects.[p12],[p13] As another example, the cardiotoxic effect in zebrafish larvae was similar to human ventricular arrhythmia following treatment with free thioridazine, which could be exploited to test the effect of alternative thioridazine formulations.[p14]

The main readout for toxicity in zebrafish is behavioral and morphological evaluation. This is because larval transparency allows for the observation of quantitative reduction of fish heart rate, malformations related to developmental toxicity, or neurotoxicity.[p15],[p16] Aspatwar *et al.* described a rapid and efficient method for screening the safety and toxicity of chemical compounds using zebrafish embryos from 1 to 5 days post fertilization (dpf). Fourteen compounds belonging to the monothiocarbamate and dithiocarbamate series were evaluated for mortality, phenotypic changes, and the half maximal lethal concentration ($LC_{50}$) dose, determined at the end of the exposure.[p17] In addition to morphological evaluation, transcriptome analysis was useful for assessing other parameters, providing further understanding of how clofazimine induces cardiac dysfunction.[p18]

## Zebrafish infected with *Mycobacterium marinum* as a model for TB

Animal models are indispensable for improving understanding of *Mtb* disease pathogenesis and for drug discovery. Unfortunately, most currently available animal models of *Mtb* infection do not completely recapitulate human TB.[p19] For example, *Mtb*-infected mice is one of the most widely used models in TB research. Modeling TB in mice has several advantages: they are small and cost-effective, and numerous mouse-specific immunological reagents are available, but the human infection has markedly distinct features, especially in relation to granuloma formation.[p20] Moreover, given that *Mtb* is not a natural pathogen of mice, rodents are more resistant to TB infection and display alternative lung pathologies.[p21] Nevertheless, mouse experiments remain the gold standard for TB research and, therefore, are required before clinical trials in humans.[p19] Although nonhuman primate animal models, such as the macaque, successfully replicate human TB infection, the high maintenance costs, limited animal availability, ethical concerns,and need for special facilities limit their general use in preclinical studies.[p22]

In addition to mice and nonhuman primates, other relevant animal models in TB research include rabbits, guinea pigs, and zebrafish. The field of TB research in zebrafish started during the early 2000s, when Davis *et al.* reported that *M, marinum* can cause a systemic infection in zebrafish larvae when injected into the caudal vein at 32 h post fertilization (hpf), with key characteristics of adult mycobacteriosis.[p23] Importantly, this landmark demonstrated that innate immunity is sufficient to initiate granuloma formation. Since then, zebrafish have become an important addition to TB research, especially because of the characteristics associated with embryonic and larval stage zebrafish research,[p24] which include low-cost maintenance, small size, and high fertilization rates,[p25] which allows testing on a high number of organisms, thus increasing statistical power potential. Zebrafish development is fast and both embryonic and larval zebrafish are visually transparent, which has promoted the creation of several fluorescent reporter lines (e.g., for blood vessels or macrophages) that enable imaging and analyses of host–pathogen interactions in real-time.[p24] Furthermore, external fertilization and a completely sequenced genome provide grounds for targeted genetic manipulation, for example with CRISPR-Cas systems. Such systems in zebrafish have long been used to further understanding of mycobacterial infection in zebrafish by the creation of mutant transgenic lines; There is also the potential to use Cas9 to generate null alleles in F0 zebrafish larva,[p26] or Cas13 to produce mRNA knockdowns,[p27] thus allowing initial assessment of the involvement of specific genes in mycobacterial infection or drug response processes without the need to raise specific lines. In addition, zebrafish up to 5 days post fertilization (dpf) are not considered laboratory animals under several animal laboratory laws, including European legislation,[p25] and, there-

fore, are accepted as a replacement for animal studies in research according to the 3Rs (Replacement, Reduction, and Refinement).[p4]

*M. marinum* is a natural pathogen of poikilothermic animals, including frogs and fish, and also the closest genetic relative and evolutional homolog of the *Mtb* complex, which causes a disease that resembles human TB.[p28] Although the orthologous coding sequences of *M. marinum* and *Mtb* have an average amino acid identity of 85%,[p24] *M. marinum* is only associated with an opportunistic skin infection in humans, allowing tests with *M. marinum* to be conducted at Biological Safety Level 2 (BSL2) instead of BSL3.[p29],[p30] Infection of zebrafish with *M. marinum* occurs naturally via the digestive tract or through abrasions in the skin.[p31] Progress has been made in developing robust and replicable systems for injecting *M. marinum* into zebrafish embryos at different stages of development. Microinjection of mycobacteria into the yolk of early developing embryos leads to a local infection that spreads to the embryo from 3 days post infection (dpi), providing an easy administration route for a consistent bacterial burden.[p24] Despite being technically more challenging, injection into the caudal vein, blood island or the posterior cardinal vein of embryos between 28 and 52 hpf, are well-established protocols to produce consistent systemic infections with a strong immune involvement that leads to spread granulomas throughout the embryo close to blood vessels. By contrast, injection into the neural tube develops a single, large, localized granuloma with consistent macrophage necrosis.[p32] Additional injection sites have been developed to study specific parameters, such as injecting in the hindbrain ventricle or the tail fin, usually devoid of immune cells, to easily visualize and asses immune cell recruitment.[p24],[p33]

One of the main interests in using this model is the ability to test drugs in a whole living animal. *In vivo* drug screening is especially important in TB because of the complexity of the disease, in which mycobacteria manipulate host defenses.[p34] Given the similar genetic and physiological makeup of zebrafish and humans, zebrafish animal models are a complementary tool to *in vitro* drug toxicity screening during the preclinical phase of anti-TB drug discovery.[p6] Overall, this model is appropriate to study TB pathogenesis, and has proved convenient for TB drug research.[p35] In that sense, the zebrafish larval model does not aim to replace murine and other gold-standard models, but to fill the gap between *in vitro* cell-based assays and more complex *in vivo* models, before committing to test in mammals.[p4],[p36] All the aforementioned advantages allow the zebrafish model to be used earlier in the drug development pipeline compared with other *in vivo* systems, even as early as hit selection, thus saving time and resources downstream. Table 2 details studies reporting anti-TB drug development using zebrafish infected with *M. marinum* model.

## Antituberculosis drug screening in zebrafish

The rise of resistant *Mtb* strains requires new drug candidates, which need *in vivo* testing after showing potential effects *in vitro*. Indeed, several compounds effective *in vitro* fail during *in vivo* assays.[p29] In this regard, one of the main advantages of the zebrafish infected with *M. marinum* model is that it can successfully be used to efficiently test antitubercular drugs *in vivo* because of recapitulation of the host infection conditions.[p37]

Initially, to show the relevance of this model to find promising compounds that deserve further studies, Habjan *et al.* tested 240 compounds that inhibited both *Mtb* and *M. marinum in vitro*, and found that only 6% were effective in zebrafish larvae infected with *M. marinum* and eligible to enter the antitubercular drug discovery pipeline.[p29] In addition, this system was used to identify and validate new therapeutic targets, which is vital to confirm the specificity of the drug effect. Zebrafish infected with a FadD32 mutant resistant *M. marinum* strain demonstrated that the effect of CCA34, a coumarin analog, resulted from inhibition of this target. This compound was demonstrated to inhibit mycobacterial proliferation in both *in vivo* models, that is, zebrafish–*M. marinum* and mice –*Mtb* infection.[p38] In general, compounds tested and active in different mycobacterial animal infection systems, such as in zebrafish and rodents, might show promise in human trials.[p37] A translational zebrafish–rodent screen reported that eight of 15 antitubercular compounds tested showed similar results between the two species, and the lack of effect in zebrafish of an additional one resulted from poor larval uptake. With good uptake and high efficacy in both zebrafish and mouse assays, GSK14 is part of a series of compounds that have progressed to extensive medicinal chemistry programs.[p37]

In some cases, *M. marinum* and *Mtb* have divergent sensitivity and resistance results in anti-TB drug screening assays. These variations were related to differences in activity and/or expression levels of some proteins between the species. Examples include prodrugs that require activation of *Mtb* enzymes that might differ in *M. marinum*. To overcome this problem, *M. marinum* strains overexpressing the mycobacterial enzymes EthA and KatG were used to discover potential new antituberculosis prodrugs, as demonstrated for ethionamide, isoniazid, and a series of antimycobacterial compounds (C1–C6) from the TB Alliance library.[p30] Similarly, specific genes from *M. marinum* with importance in the infection or drug susceptibility process can be replaced with orthologs from *Mtb*. An example is the replacement of *M. marinum* blaC for the homologous *Mtb* gene. This system has the advantage that the effect of specific mutations of interest can be tested in a context closer to the *Mtb* infection conditions than achieved using *Escherichia coli*.[p39]

Results evaluating antimycobacterial potency, host survival, and pathological effects of the compounds tested were complemented by the previously mentioned toxicity and developmental effects between different treatments.[p40] To do so, antimycobacterial and phenotypic parameters were analyzed in parallel to discover whether drugs will have enough potency for antituberculosis effects while showing no toxicity. This was shown in a study that demonstrated that the dithiocarbamate Fc14-584B did not show significant phenotypic changes and no damage in the internal tissues while decreasing the *M. marinum* burden at a specific concentration.[p6] Similarly, a bis-substituted cyclam derivative, compound 21b, identified by structure–activity relationship studies, with improved water solubility, was found to be potent and active against drug-resistant *Mtb*. After biological tests, this compound demon-

strated a reduction in bacterial load in zebrafish larvae infected with *M. marinum* without morphological effects on larval development.[p41]

Drug uptake determination is an important tool to understand and correlate drug screening results from zebrafish with other frequently used animal models: drugs that showed the most encouraging results in uptake and potency in zebrafish assays were also effective in rodent models. Zebrafish offer the possibility to test compounds in a model with numerous polymorphisms and a reduced metabolic rate. This is especially important in comparisons with the diverse human population.[p37] Habjan *et al.* used the zebrafish embryo infection model with *M. marinum*, *E. coli*, or *Streptococcus pneumoniae* to demonstrate that antibiotics that are clinically administered via intravenous or intramuscular injection and as oral drugs showed activity when injected into the zebrafish bloodstream or added to tank water, respectively. These findings suggested that waterborne treatment compounds had an enhanced chance of good oral uptake.[p29] While zebrafish pharmacology is still in its infancy, in particular regarding initial developmental stages, it is possible to evaluate the pharmacokinetic–pharmacodynamic parameters (e.g., clearance, $E_{max}$, and volume of distribution) of drugs in larvae and translate the response to humans using nanoscale blood sampling and nondestructive quantification of bacterial burden by fluorescence microscopy. This method confirmed the translational response of isoniazid in zebrafish to humans.[p25]

## High-throughput anti-TB drug discovery

The combination of a larval small size and easy manipulation together with recapitulation of mycobacterial infection with fluorescence or luminescence-based readouts makes zebrafish amenable to high-throughput screening (HTS) strategies to screen numerous compounds for antibacterial activities.[p29],[p42] Although multicellular models for TB drug discovery have been developed by creating *in vitro* granulomas, they do not represent the features of a whole organism.[p43] Moreover, strategies of HTS using *Mtb* are time-consuming and expensive because of the need for BSL3 facilities.[p30] Other *in vivo* models, such as adult zebrafish, rodents, and primates, are ethically questionable as HTS models. Compounds tested *in vitro* against *Mtb* had consistent results in terms of bioluminescence decreases in zebrafish infected with *M. marinum*, demonstrating that it is an effective model for *in vivo* TB drug screening.[p44]

Additional increase on throughput is being tackled by the robotic microinjection of zebrafish embryos, coupled with automatic screening using high-resolution imaging after treatment using large particle flow cytometers.[p42] Carvalho *et al.* showed that it is possible and effective to couple the microinjector to a flow cytometer (complex object parametric analyzer and sorter, COPAS) and rapidly test the efficacy of anti-TB compounds.[p45] The bacterial burden in live larvae infected with fluorescent *M. marinum* was quantified by two rapid methods: fluorescence quantification of images via flow cytometry, or by automated plate fluorimetry, in which a 96-well plate was loaded into a fluorescence plate reader and bacterial fluorescence measured and normalized to larval autofluorescence. Thus, this high-throughput method is able to evaluate the efficacy and toxicity of drugs and new antibacterial agents.[p34],[p33]

## Beyond antibiotic drug screening: alternative approaches to anti-TB drug discovery

In addition to discovering traditional new antibiotics, the use of the zebrafish larval infection model has been applied to other fronts of anti-TB drug discovery, such as novel approaches to increase the efficacy of current therapies using NPs as drug delivery, or alternative drug actions, such as host-directed therapies (HDTs).

### *Improving drug delivery and nanoparticles as drug delivery systems*

One of the main focus of the pharmaceutic industry is not to discover new bioactive molecules per se, but to adequately deliver them to the target tissue in an effective, nontoxic way. to this end, nanotechnology has been used to overcome some challenges in anti-TB drug delivery. Polymer–drug conjugation offers an alternative to increase drug solubility, intensify the therapeutic effectiveness, control drug release, and also reduce side and toxic effects.[p36],[p46] The zebrafish larval model has been extensively used to monitor the persistence and stability of NPs in the blood circulation, interactions with the host immune cells, and its localization in granuloma structures.[p4]

NP-encapsulated drugs were more potent compared with free drugs in decreasing the bacterial burden and increasing the survival of embryos. For example, some NP formulations were specifically taken up by macrophages, thus allowing specific targeting of the mycobacterial niche, which was followed in real time in fluorescently labeled immune cells in transparent zebrafish larvae. This was exemplified by a study in which rifampicin encapsulated in NPs, made from a biodegradable polymer [poly (lactic-*co*-glycolic) acid; PLGA], were phagocytosed by macrophages and delivered to the *M. marinum* granulomas, showing higher effectiveness c free diffused rifampicin.[p47] The pretomanid derivative, micellar formulation D, was found to interact with granulomas in zebrafish larvae and showed consistent results in the *in vitro* inhibition of *Mtb* and *M. marinum*, as well as in the *in vivo* activity of zebrafish infected with *M. marinum* and mice infected with *Mtb*.[p48] As an additional targeting requirement, some NPs can only be degraded in the phagolysosomes of macrophages, targeting drug release in specific cells and reducing systemic toxicity.[p14] Zebrafish infected with fluorescent *M. marinum* and treated with alginate-capped silver NPs (ALG-AgNPs) showed a decrease in fluorescence signal at nontoxic doses, thus inducing a strong antimycobacterial potential in zebrafish that was later confirmed in a mouse TB model.[p49]

Similarly, Trousil *et al.* developed five biodegradable and biocompatible rifampicin nanoformulations and tested the most promising in zebrafish infected with *M. marinum*. Results showed that rifampicin-loaded NPs increased efficacy and larval survival compared with free rifampicin, and empty NPs were also well tolerated by zebrafish larvae.[p46] A similar effect was found for rifampicin-encapsulated polymersomes, which required a lower dosage to reduce *M. marinum* burden *in vivo* compared with the same concentration of free drug.[p50] Finally, all of these approaches are amenable for combination therapy with multiple

drugs. Infected zebrafish treated with isoniazid and clofazimine nanobiotics showed better results, in terms of the reduction in bacterial burden and granuloma number, compared with controls and free drugs.[p36]

### *Host-directed therapy*

HDTs have been proposed as relevant adjunct therapeutic strategies against TB to overcome antimicrobial resistance, modulating the host immune system instead of targeting pathogens.[p51] While cell culture settings have been used for the study of potential host-directed drugs, multicellular interactions and the complex environment involved in mycobacterial *in vivo* disease are difficult to capture *in vitro*.[p43] Zebrafish larvae have become a far-reaching *in vivo* model to produce a more meaningful HDT for TB research,[p24] because they not only allow the readout of a potential effect on mycobacterial burden, but also aid study of the host mechanism responsible for mycobacterial death following a drug mode of action.

For example, Matty *et al.* used zebrafish larvae infected with fluorescent *M. marinum*, to screen 1200 FDA-approved drugs for the identification of novel HDTs with antimycobacterial activity, ultimately finding that clemastine enhances macrophage-induced mycobacterial killing *in vivo*.[p43] This infection model was also used to identify that the cyclic nitroxide antioxidant MetT decreases bacterial burden directly, as well as via the host inhibiting mitochondrial reactive oxygen species (mROS) production and bacterial spread by reducing cell death in the mycobacterial granuloma.[p35] Pretreatment with the histone deacetylase (HDAC) inhibitors, Trichostatin A and TMP195, decreased *M. marinum* burden in infected zebrafish embryos, supporting the results of *in vitro* human macrophages assays and the efficacy of these inhibitors in early stages of granuloma formation.[p52]

The advantage of using a whole *in vivo* system can also provide novel insights into potential synergies between different cell types or organs. Previous studies found that mycobacterial infection activates signaling of the ligand-activated transcription factor aryl hydrocarbon receptor (AhR) *in vivo* including in mouse models; AhR is a well-known activator of among others, xenobiotic metabolism. Infected zebrafish cotreated with rifabutin and CH-223191, an AhR inhibitor, showed enhanced rifabutin-mediated *M. marinum* killing compared with controls, corresponding with an elevated anti-TB drug concentration.[p53]

Finally, the ability to follow and visualize the infection dynamics in real-time is valuable to not only discover new HDTs, but also understand their mechanisms of action. Roca *et al.* used zebrafish to discover the mechanism by which TNF-induced mROS are involved in the pathogenesis of mycobacterial infections, leading to the use of metformin as an inhibitor of this process and in the treatment of TB.[p54] Recently, Boland *et al.* visualized *in vivo* how tamoxifen increased the autophagy flux in the host, thus having a beneficial effect in clearing TB infection.[p51]

## Concluding remarks

We have showcased the relevance and versatility of zebrafish embryonic and larval models to improve and discover new drugs for the treatment of TB. We highlighted research that tested anti-TB compounds in zebrafish and mice models, demonstrating similar drug-intrinsic properties and *Mycobacterium* growth inhibition. Given the high similarity in pathogenesis between *M. marinum*- and *Mtb*-infected hosts, easy genetic manipulation, and the possibility to track host–pathogen interactions and pharmacological activity in real time, zebrafish have become a valuable tool in TB research.

However, like all animal models, the models highlighted in this review have limitations, both intrinsically related to the model itself and because of technological limitations. For example, the optimal growth temperature of zebrafish is 28 °C, limiting the potential use of *Mtb* as an infecting agent. Although *M. marinum* can produce equivalent infection in zebrafish to *Mtb* in primates and humans, there is an inherent risk of different susceptibilities between mycobacteria. This limitation is partially solved by the traditional pipeline of *in vivo* studies positioned after *in vitro* analysis (which can be performed with *Mtb*), as well as with the use of transgenic *M. marinum* strains with key enzymes from *Mtb*, as we have discussed. Similarly, the use of embryonic and larval models, where the advantages for drug discovery are the greatest, supposes that only innate immunity (i.e., macrophages and neutrophils) is present, thus making the study of processes that involve the adaptive immune system difficult, or assuming that some organs, such as the liver, will only be active at later stages. By contrast, drugs with antimycobacterial effects in zebrafish have a greater probability of being effective in patients who are immunocompromised. Other limitations of the model can be solved via careful experimental design or are in development. For example, compounds with poor water solubility will have to be studied via direct compound injection, which can be technically challenging. To solve this limitation, the development of robotic injection methods holds great promise. Other current efforts include the creation of 'humanized' zebrafish lines and the development of pharmacokinetic models, which suggest a strong translational potential between zebrafish and mammals via allometric scaling.

Zebrafish models have long added to our understanding of infection biology and the host–pathogen interaction, but with the first drugs initially discovered in zebrafish reaching clinical trials (e.g., ProHema for graft-versus-host disease; clemizole for Dravet syndrome, among others),[p55] zebrafish modeling is starting to show translational potential for drug discovery against human diseases and is primed to be integrated into the TB drug discovery pipeline.

## CRediT authorship contribution statement

**Stella S. Antunes:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Gabriel Forn-Cuní:** Writing – original draft, Data curation. **Nelilma C. Romeiro:** Writing – review & editing. **Herman P. Spaink:** Writing – review & editing, Project administration, Funding acquisition. **Fons J. Verbeek:** Writing – review & editing, Project administration, Funding acquisition. **Michelle F. Muzitano:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

## Data availability

No data was used for the research described in the article.

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