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## **Boosting the host immune system to fight tuberculosis**

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# 1

## **Introduction and outline of this thesis**

## Tuberculosis

Tuberculosis (TB) is an increasing global health problem. This infectious disease is ranked as the leading cause of death from a single bacterial infectious agent: *Mycobacterium tuberculosis* (*Mtb*). TB often manifests clinically as a lung infection but it is also common in extrapulmonary forms, such as skeletal and lymphatic infections, meningitis, and miliary TB, which spreads to multiple organs. Characteristic symptoms include coughing, fever, chronic fatigue, and severe weight loss. Globally, 10 million people developed TB and 1.4 million died from the disease in 2019<sup>1</sup>. Furthermore, it is estimated that one third of the global population carries a latent *Mtb* infection, characterized as a clinical state without evidence of disease, but with a positive reaction to the tuberculin skin test. Latent infections can lead to active disease, especially if the host is immune compromised, for example due to HIV infection. Of all people infected with HIV who died in 2019, one third were infected with *Mtb* as well<sup>1</sup>.

Current treatment of TB consists of daily doses of first-line antibiotics (isoniazid, rifampicin, ethambutol and pyrazinamide) for six months. In some cases the bacteria are resistant to these first-line antibiotics, and subsequently patients need to be treated with second-line antibiotics that have more side effects and are more costly (i.e. bedaquiline, delamanid, lefloxacin and moxifloxacin)<sup>1,2</sup>. Furthermore, the treatment of latent *Mtb* infection is complicated, because bacteria are dormant and antibiotics disrupting bacterial cell-wall synthesis or other bacterial cell-cycle components are hardly effective in non-dividing bacteria. In addition, the currently used BCG-vaccine, which is a century old, only offers partial protection against TB. While a dozen clinical trials for new vaccines are taking place, an effective vaccine against TB is yet to be developed<sup>3-6</sup>.

Developed countries with high standards of living and adequate healthcare systems have eradicated active TB almost completely. However, the rise of multi-drug resistant (MDR) and extensively-drug resistant (XDR) *Mtb* strains is cause for concern. It is believed that poor adherence of patients to first-line antibiotic treatment regimens works in favor of the pathogen developing resistance<sup>7,8</sup>. As conventional treatments become less effective, the threat of TB is becoming larger not just in developing countries, but in countries with better healthcare systems as well. For instance, despite that the overall TB disease burden in the Russian Federation is falling, the incidence of MDR-TB is rising. Moreover, while Europe accounts for only 2,5% of the global disease burden, 17% of new cases in Europe were MDR-TB. Globally, almost half a million TB infections were due to MDR *Mtb* strains in 2019<sup>1</sup>.

Ending the epidemic of TB by 2030 is one of the United Nations sustainable development goals<sup>1</sup>. To achieve this ambitious goal, scientists around the world are investigating the disease, the pathogen *Mtb*, and the interaction between the bacterium and its human host. In addition, diverse animal hosts are used to model different aspects of TB<sup>9</sup>. New insights into the disease and host processes involved in the disease are used to find new treatment options. While we aim to fight *Mtb* with new therapeutic strategies, *Mtb* itself has many tricks up its sleeve that make it such a successful pathogen.

## Subversion of the immune system

Upon infection, *Mtb* is quickly phagocytosed by professional phagocytes, especially macrophages. Phagocytosed *Mtb* are contained in phagosomes that have to fuse with lysosomes for acidification and degradation of their contents. During the process of phagosome maturation and phagosome-lysosome fusion, bacteria are exposed to a variety of host-defense mechanisms, such as proteases, antimicrobial peptides,

and reactive nitrogen and oxygen species<sup>10-12</sup>. However, *Mtb* and other pathogenic *Mycobacteria* have the remarkable capability of arresting phagosome-lysosome fusion via excreted virulence factors as well as cell envelope components<sup>13,14</sup>. In addition, *Mycobacteria* have evolved mechanisms to protect themselves against phagosomal and lysosomal killing mechanisms<sup>15-17</sup>. Subsequently, they are able to replicate within these vesicles and eventually permeabilize them to escape into the cytosol<sup>18,19</sup>. Escaped cytosolic bacteria or arrested phagosomes can be targeted for autophagy, an intracellular degradation pathway vital to maintaining homeostasis. Via the autophagic pathway, unwanted elements, such as protein aggregates, damaged organelles but also intracellular bacteria, are removed from the cell<sup>20-22</sup>. However, like other host defense mechanisms, also autophagy is inhibited by *Mtb* to some extent<sup>23</sup>.

The intracellular presence of *Mtb* causes macrophages to form aggregates, which initiates the formation of tuberculous granulomas. Granulomas are the pathological hallmark of TB and consist of a core of infected macrophages and necrotic cell debris, and a wall of several cell layers that contains various cell types, such as neutrophils, dendritic cells and T- and B-cells<sup>24</sup>. It was long believed that granulomas serve strictly a host-protective function and that granuloma formation represents a host strategy to contain *Mtb* infection. However, this view has been challenged by the findings that *Mycobacteria* actively promote granuloma formation and that directed aggregation of macrophages by *Mycobacterial* virulence factors facilitates dissemination of the bacteria in the infected host<sup>24-26</sup>. These results have shown that *Mycobacteria* benefit from granuloma formation during the early stages of infection. Nevertheless, it is important for the infected host to maintain the structure of mature granulomas, as active TB develops under conditions where granuloma integrity is compromised. For example, this can occur during HIV infection or in patients receiving anti-inflammatory therapy with TNF blockers. It is because of this dual role of granulomas in TB and the intricate interplay between *Mtb* and host-immune-related processes that new therapeutic strategies are desperately needed.

### Host-directed therapeutics

Most antibiotic targets are either components of the bacterial cell-wall or involved in cell-wall synthesis<sup>9</sup>. It is believed that resistance to a specific antibiotic can lead to faster developing resistance against other antibiotics with similar targets. The search for new antibiotics continues as more and more pathogens become resistant<sup>27,28</sup>. New antibiotics are sporadically discovered<sup>29</sup>, however they are often used as a last-resort, to prevent the rise of resistance against these new antibiotics. Efforts from pharma companies to find new antibiotics are therefore limited, as they are not profitable, in part because of this last-resort policy<sup>30</sup>.

Contrary to antibiotics that are directed against the pathogen, host-directed therapeutics (HDTs) aim to modulate host-pathways to potentiate the host-immune response against pathogens such as *Mtb*<sup>31-35</sup>. This can be achieved in several ways: first, HDTs can improve the bactericidal capacity of immune cells. Second, HDTs can limit detrimental effects of inflammation. Third, HDTs can overcome suppressed immune responses by *Mtb* or elicit novel immune responses against *Mtb*. And fourth, HDTs can target host factors that are manipulated by *Mtb* for its own pathogenesis. By enhancing host defense, HDTs have the potential to shorten treatment regimens with conventional first-line antibiotics<sup>36,37</sup>. Importantly, for some HDT candidates, for example imatinib and H89, it was shown that they are effective against antibiotic-resistant *Mycobacteria* offering a possible answer to the rise of MDR and XDR *Mtb* strains<sup>36,38</sup>. It is expected that because HDTs do not directly target bacteria, resistance is less likely to develop<sup>35</sup>.

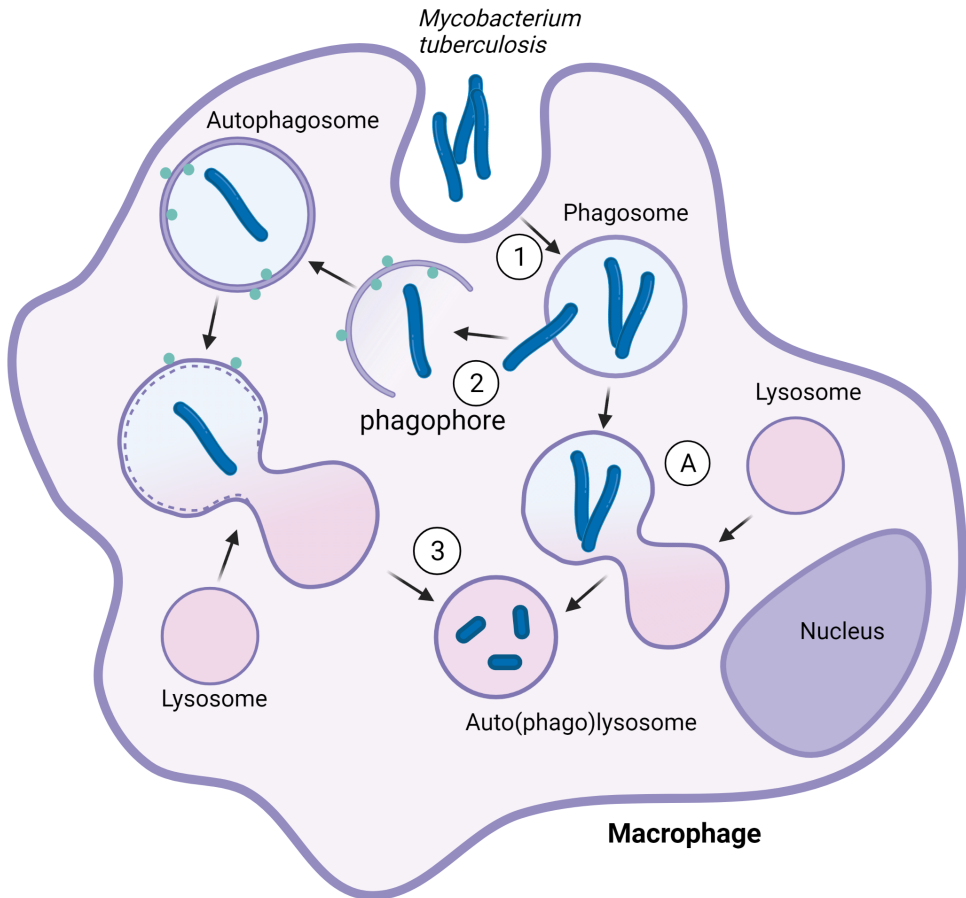
In the search for HDTs for TB and other infectious diseases, drug repurposing screens are often employed. The principle behind drug repurposing is that drugs that have been approved for clinical use may have additional effects besides those for which they are registered, and therefore these drugs may be utilized for other therapeutic applications. Similarly, there is a large unexplored potential in candidate drugs that did not pass phase-II clinical trials for efficacy assessment but may prove effective in other disease treatments than the one they were originally tested for. One advantage of drug repurposing compared to the development of novel HDTs, is that most of these compounds have already passed phase-I clinical trials for safety assessment. When these compounds are proven to work in animal models for TB, they could potentially be tested immediately in phase-II trials and the development time for new therapies is greatly reduced.

In recent years, several laboratories have reported on results of large-scale screening of compound libraries and genetic targets, in which many potential candidate HDTs for TB treatment have been identified<sup>38-42</sup>. The majority of these screens are performed *in vitro* with cultured cells or monocyte derived macrophages, but they also can be performed using a suitable *in vivo* model, such as the zebrafish model for TB<sup>43</sup>. Several excellent reviews have described the current status of HDT identification for TB<sup>31,33,35,44</sup>. In this chapter we highlight HDT strategies that focus on autophagy and (auto)phagolysosomal pathways. In addition, we discuss how the zebrafish model can contribute to HDT screening and be used to translate *in vitro* effects of HDTs to a straightforward *in vivo* model of TB.

## Autophagy

The most common arm of the autophagy pathway is called macroautophagy (hitherto autophagy) and describes the clearance of intracellular waste or cargo, such as organelles, lipids and proteins via autophagosomes (Figure 1). These double membraned compartments fuse with lysosomes to form autolysosomes in which the cargo is digested into cellular building blocks such as fatty acids and amino acids<sup>45</sup>. At the beginning of this century, it was reported that induction of autophagy in macrophages leads to protection against *Mtb*<sup>20,46</sup>. It has now become well established that autophagy plays an important role in the clearance of intracellular bacteria and other microbes. First, the induction of bulk, or non-specific, autophagy by starvation or by inhibition of mTOR (mammalian target of rapamycin) signaling can lead to increased intracellular bacterial killing<sup>21</sup>. In addition, autophagy can also reduce bacterial growth because it limits inflammation<sup>21</sup> and promotes antigen presentation to T-cells<sup>47</sup>.

Recent studies have highlighted that autophagy often occurs as a selective, receptor-mediated process<sup>48,49</sup>. Selective autophagy is classified depending on the cytoplasmic material that it targets. For example, xenophagy targets microbes, mitophagy targets damaged mitochondria, and aggrephagy targets protein aggregates<sup>50,51</sup>. Specific receptors mediate selective autophagy by linking the cargo directly to the microtubule-associated light chain 3 protein (LC3), which is conjugated to the membrane of nascent and mature autophagosomes<sup>48</sup>. In the case of xenophagy, microbes that have escaped the phagosome are ubiquitinated and recognized by members of the Sequestosome (p62/SQSTM1)-like receptor (SLR) family, a family of selective autophagy receptors that includes p62, NDP52, NBR1, TAX1BP1, and OPTN (optineurin). Xenophagy has been well established as an important effector of innate immunity. For instance, p62 and optineurin have been shown to be required for the autophagic defense against mycobacterial infection in the zebrafish model for TB<sup>52</sup>. Furthermore, it is believed that the generation of neo-antimicrobial peptides, which are effective in killing *Mtb*, is



**Figure 1. Role of the autophagic pathway in *Mycobacterium tuberculosis* clearance**

*Mtb* is phagocytosed (1) and contained in a phagosome from which it can escape (2). Following phagosomal escape, the bacteria are targeted to the autophagic pathway via LC3 (green dots). After the bacteria is contained in an autophagosome, the autophagosome fuses with a lysosome and the contents is degraded. Alternatively, bacteria can remain contained in the phagosome, which fuses with a lysosome (A) after which the contents is degraded.

mediated by ubiquitination and delivery of proteins to microbe-containing compartments by p62 and related receptors<sup>53</sup>.

Although autophagy is recognized as an important host-protective pathway<sup>21,22</sup>, the interplay between *Mtb* and autophagy is complex. A recent study showed no effect on susceptibility to *Mtb* in mice with mutations in different autophagy proteins and has therefore questioned the role of autophagy in the immune response to *Mtb*<sup>54</sup>. In this study, only a mutation in ATG5 led to increased susceptibility to *Mtb*, confirming a previously shown antimycobacterial effect of this autophagy protein<sup>55</sup>. However, Kimmey *et al* did not attribute this antimycobacterial effect to the role of ATG5 in autophagy, but to the prevention of an immuno-pathological neutrophil response via ATG5. Furthermore, they did not see an increase in susceptibility in p62 loss-of-function mutants, which is in contrast with other studies<sup>52,56</sup>. Together, these studies suggests that the impact of autophagy on infection outcome depends critically on experimental conditions. The complexity of the interplay between *Mtb* and autophagy is further demonstrated by the

ability of *Mtb* to inhibit LC3-associated phagocytosis (LAP)<sup>57</sup>, which is an autophagy-related process contributing to host defence<sup>58</sup>. During LAP, the phagosome membrane is directly decorated with LC3 resulting in fusion of the so-called LAPosomes with lysosomes. However, *Mtb* is well known for its capability to evade immune defences, including autophagy and LAP, which could also explain why autophagy mutations had limited effect on susceptibility in some studies<sup>54</sup>. Boosting autophagy levels using HDTs could be a way to overcome the pathogen's autophagy evasion strategies and could therefore be a promising therapeutic route<sup>23</sup>.

### HDTs strategies to boost autophagy and lysosomal degradation

One of the best-known autophagy modulating drugs is Rapamycin, which can induce autophagy by inhibiting the negative autophagy regulator mTOR. However, rapamycin has properties beyond autophagy induction and it is used as an immuno-suppressive drug during organ transplants<sup>59</sup>. Due to its immuno-suppressive effects, Rapamycin is not well suited for clinical use against TB, although targeted delivery to macrophages may be considered<sup>60</sup>. Furthermore, Rapamycin is metabolized by CYP3A4<sup>61</sup>, a hepatic enzyme that is greatly induced by the antibiotic Rifampicin, which is an important first-line drug used in TB treatment. In the zebrafish model for TB, inducing autophagy using Rapamycin was also shown to be detrimental for the defense against mycobacteria, presumably due to its immunosuppressive effects or due to toxic side effects<sup>62</sup>. Similarly, mTOR inhibition by molecules related to Rapamycin might be ineffective. In fact, the small-molecule inhibitor Torin 1 increased susceptibility to *Mtb* infection in human macrophages, most likely due to reduction of phagosome acidification which led to increased *Mtb* replication<sup>63</sup>. In contrast, the mTOR inhibitor Everolimus showed promising results in a study using an *in vitro* TB granuloma model<sup>64</sup>. Single-drug treatment using Everolimus increased levels of autophagy and decreased *Mtb* burden and oxidative stress. In addition, Everolimus was also effective in a combinatorial treatment regime with the antibiotics, Isoniazid and Pyrazinamide, important first-line drugs used in TB treatment.

Autophagy can also be induced by drugs acting on signaling molecules upstream of mTOR. A promising drug for TB treatment is Metformin, which promotes the expression of the energy sensor AMP-activated protein kinase (AMPK), resulting in inhibition of mTOR. Metformin is used in the treatment of adult-onset diabetes. Of note, diabetes is known to increase the risk of developing TB as well as complicating its treatment<sup>65</sup>. Therefore, the antimycobacterial effect of Metformin is particularly relevant. Metformin was shown to be able to increase phagolysosome fusion as well as mitochondrial ROS production, thereby inhibiting *Mtb* growth *in vitro*<sup>37</sup>. Combinatorial treatment of Metformin and the first-line antibiotic Isoniazid showed a minor, but significant, inhibition on mycobacterial burden as compared to Isoniazid alone. Furthermore, Metformin treatment decreased the inflammatory response, thus reducing negative effects of inflammation such as tissue damage. Metformin was also found to enhance the adaptive immunity response to mycobacterial infection<sup>66</sup>.

A number of other autophagy modulating drugs have emerged from high-content and high-throughput screens of small molecules. Using a microscopy-based assay, Stanley et al. identified Gefitinib to induce autophagy and inhibit *Mtb* in macrophages<sup>67</sup>. Gefitinib is an anti-cancer epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor and induces autophagy potentially in an EGFR unrelated manner<sup>68</sup>. Not only did Gefitinib reduce *Mtb* in human cultured macrophages, it also reduced bacterial replication in a murine model for TB. The same study identified Fluoxetine as an antimycobacterial compound. Fluoxetine is a selective serotone reuptake inhibitor and is widely known as an



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anti-depressant under the name Prozac. Treatment of *Mtb* infected human macrophages led to a significant increase in TNF- $\alpha$  and induction of autophagy. TNF- $\alpha$  induction can indeed induce autophagy and is highly relevant for the immune response to numerous bacterial infections, including TB<sup>69</sup>. Interestingly, the anti-psychotic drugs Haloperidol, Nortriptyline and Prochlorperazine have all been shown to induce *in vitro* killing of *Mtb*<sup>70</sup>. Prochlorperazine and Nortriptyline activate autophagy via mTOR inhibition, while the same study showed Haloperidol to enhance endosomal progression. While other underlying mechanisms could be at play, the induction of autophagic degradation by these anti-psychotic drugs could be a common explanation for their effect against *Mtb*.

Anticonvulsant drugs, including Carbamazepine and Valproic acid, are another class of anti-TB compounds revealed by drug screening<sup>41</sup>. Among these drugs, Carbamazepine was shown to stimulate autophagy and decrease intracellular mycobacteria in both *in vitro* and *in vivo*, using macrophages, zebrafish, and mice models of TB<sup>41</sup>. Carbamazepine induces autophagy independently of mTOR by reducing myo-inositol uptake by macrophages, inducing autophagy through increased phosphorylation of AMP kinase and ULK1

Kinases are among the most frequently used drug targets in general and are also explored as HDTs for TB. The tyrosine kinase inhibitor, Imatinib, is used as a therapeutic in cancer treatment and has been shown to reduce *Mtb* burden by promoting phagolysosomal processes<sup>36</sup>. Mechanistically, Imatinib inhibits tyrosine kinases ABL1 and ABL2 and ABL family tyrosine kinases can regulate autophagy. Napier *et al* showed that Imatinib treatment leads to reduced bacterial burden, increased acidification of vesicles and increased percentages of mycobacteria in lysosomes. Furthermore, the AKT1 kinase inhibitor H89 has been shown to be effective in inducing phagosomal maturation to phagolysosomes and reducing intracellular bacterial growth of both *Salmonella* and *Mtb*<sup>38</sup>.

Finally, an interesting class of drugs that mediate phagolysosomal degradation are statins, clinically used to reduce cholesterol levels. Paradoxically, statins can inhibit phagosomal acidification, which is expected to prolong survival of *Mtb*. However, statins are also found to prevent phagosome escape by *Mtb*, thereby increasing (auto)phagolysosomes containing *Mtb* and promoting bacterial degradation<sup>71-73</sup>. Increasing autophagy or enhancing lysosomal processes are closely related drug effects, which we also show in chapters 4 and 5, where Tamoxifen and Amiodarone, besides increasing autophagy, also increase (auto)phagolysosomal processes. Figure 2 contains an overview of the above described HDTs and how they function.

### **The zebrafish model for tuberculosis and autophagy research**

The zebrafish (*Danio rerio*) is a small sub-tropical fish originating from south-east Asia<sup>74</sup>. It has become widely used as a model animal with its roots in developmental research<sup>75</sup>. Since the early 2000s its potential as a vertebrate model in biomedical research became apparent<sup>76,77</sup>. Today, the zebrafish model is an invaluable addition for disease and translational biomedical research as an intermediate between *in vitro* models and mammalian animal models<sup>78</sup>. Zebrafish are highly suitable for this purpose as they possess several distinct qualities beneficial for biomedical research. First, they are optically transparent in early embryonic and larval stages. This is ideal for imaging using fluorescent microscopy and confocal microscopy to gain biomolecular insights that could not have been achieved using adult animals (Figure 3), even to the point where correlative light and electron microscopy is possible<sup>77,79</sup>. Second, genes are highly conserved between zebrafish and humans, especially those associated

### HDTs that stimulate autophagy

Everolimus  
Metformin  
Gefitinib  
Fluoxetine  
Haloperidol  
Nortriptyline  
Prochlorperazine  
Carbamazepine  
Tamoxifen  
Amiodarone

### HDTs that inhibit phagosomal escape

Statins

### HDTs that promote phagosome maturation

Imatinib  
H89

### HDTs that stimulate lysosome formation

Tamoxifen  
Amiodarone

### HDTs that increase lysosomal degradation

Imatinib

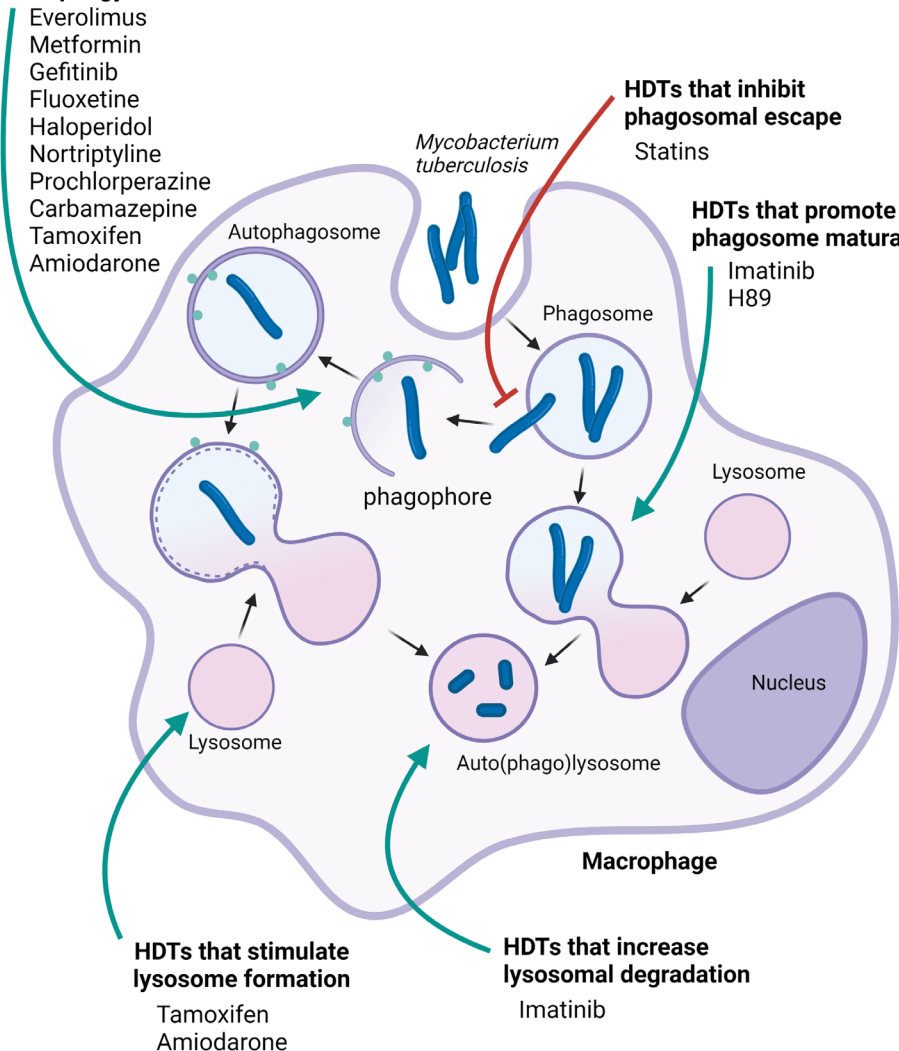
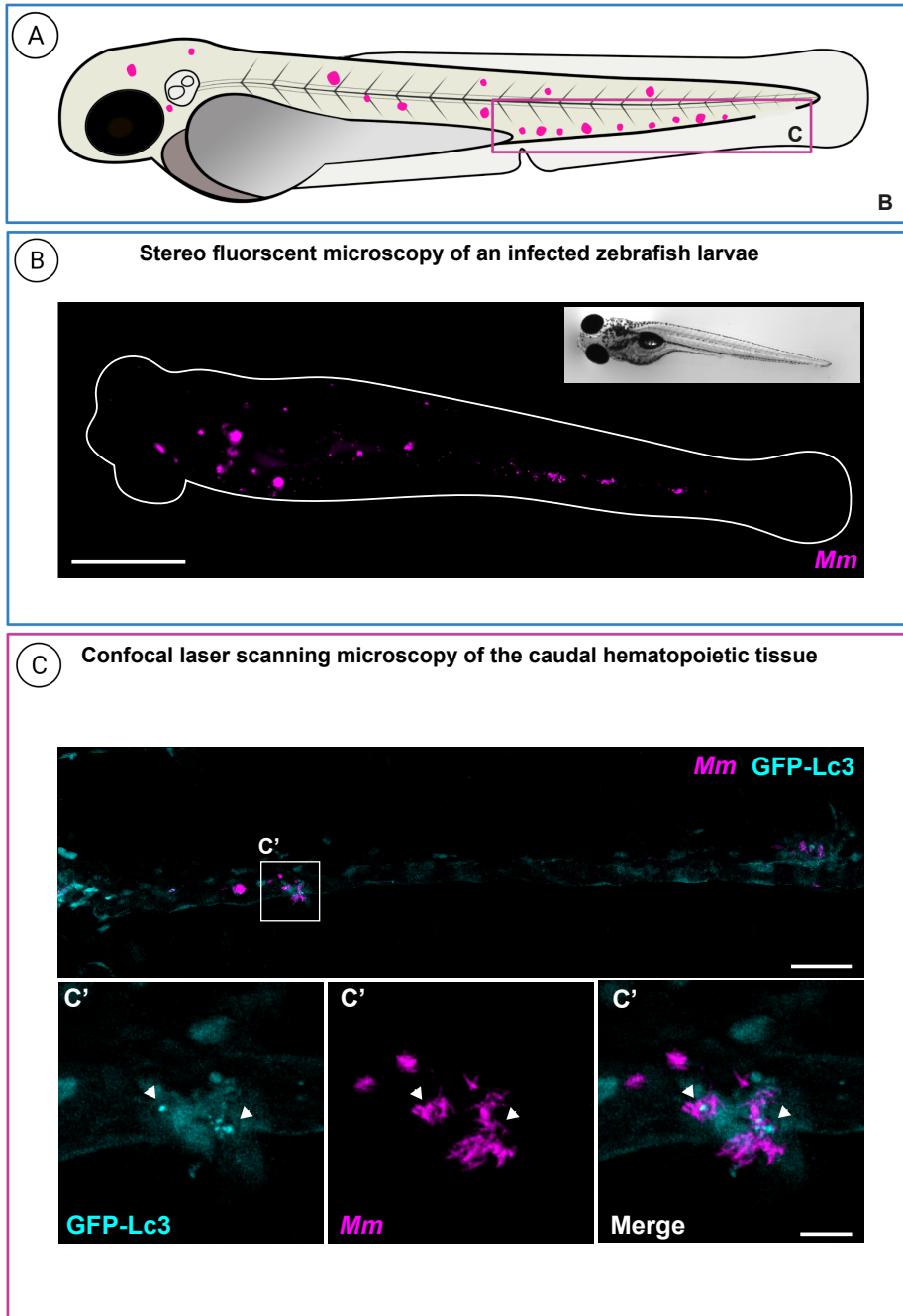


Figure 2. Overview of HDTs and how they modulate the autophagic-, phagosomal- and lysosomal-pathway. Green lines denote a stimulating effect while the red line denotes an inhibiting effect.

with disease phenotypes where 84% of human genes have identified counterpart in zebrafish<sup>80</sup>. Third, because of external fertilization, genetic modification can be easily performed by injecting DNA constructs or knockdown/knockout reagents into the zebrafish eggs at the one-cell stage, and precise genome editing has become even more straight-forward with CRISPR/Cas9 techniques<sup>78,81,82</sup>. As a result, a wide variety of knock-out and reporter lines are available in the zebrafish research community. Fourth, zebrafish are relatively easy to maintain compared to mammalian models and they take up far less space, making it also an economically interesting model<sup>77</sup>. Fifth, innate and adaptive immunity are separated in development by 2 to 3 weeks, making it possible to study host-pathogen interactions exclusively during the innate immune response in zebrafish embryos and larvae<sup>83,84</sup>. Sixth, zebrafish embryos and larvae are especially



**Figure 3. Examples of two imaging techniques using the zebrafish model**

- A. Representation of an infected zebrafish larva, 5 days post fertilization. Magenta dots indicate *Mm* clusters.
- B. Example of stereo fluorescent image of whole larvae infected with mWasabi-expressing *Mm*. Magenta shows *Mm*. Scale bar annotates 1 mm.
- C. Example of confocal microscopy max projection images of the caudal hematopoietic tissue (CHT) region of an infected transgenic GFP-Lc3 zebrafish larvae. Cyan shows GFP-Lc3 positive vesicles and magenta shows *Mm*. Scale bar annotates 50  $\mu$ m.

suitable for screening drugs, which can be easily administered via the water and are taken up through the skin<sup>85</sup>. Finally, adult zebrafish share physiology and anatomy with vertebrates, including humans, and many processes in disease are similar to that in humans<sup>78</sup>. All these advantages make the zebrafish an attractive model animal to study mechanisms of disease, metabolic disorders, genetic disorders, cancer, infections, behaviour, and to apply zebrafish in drug discovery pipelines.

Infection of zebrafish with the natural fish pathogen *Mycobacterium marinum* (*Mm*), a close relative of *Mtb*, leads to pathogenesis remarkably similar to TB-pathogenesis<sup>24,86,87</sup>. Using the zebrafish model for TB, important insights have been obtained for example on the role of granulomas that are characteristic for TB pathology. Though it was long thought that the granulomatous aggregates of leukocytes are mainly a host defence structure, encapsulating the bacteria, it was the zebrafish model that provided evidence that these aggregates are dynamic structures that aid dissemination of bacteria, especially at the early stages of their development that can be visualized in zebrafish larvae<sup>26</sup>. Important virulence factors, including those that promote granuloma formation, are similar in *Mtb* and *Mm*<sup>25,88</sup>. Adult zebrafish can be used as a TB model by intraperitoneal injection of *Mm* and they can be used to model latent TB disease, overcoming an important limitation of other TB animal models<sup>89,90</sup>. However, the versatility and possibilities of the embryonic and larval stages are the biggest contributors to the popularity of zebrafish as a vertebrate model for TB<sup>87</sup>. Experimentally, embryos can be injected with bacteria as early as 1 day post fertilization and both systemic or local infections can be achieved using micro-injection techniques<sup>91</sup>. Because there is no need for feeding during the first week of development and development is normal even under anesthesia, the embryos and larvae are ideal for non-invasive imaging. The zebrafish-*Mm* model has therefore proven highly useful to study host-pathogen dynamics during the early stages of infection using specific phagocyte-lineage reporter lines<sup>92-95</sup>. Of particular interest for this thesis is the use of the zebrafish embryo model for autophagy research in the context of TB.

Zebrafish have been used to study autophagy in the context of development and disease, including infection. Using both genetic knockdown of autophagy genes and chemical modulation of autophagy, using commonly used autophagy inhibitors or inducers, zebrafish have helped elucidate the role autophagy machinery in various developmental and disease contexts<sup>96-99</sup>. To study anti-mycobacterial autophagy *in vivo* we have used the zebrafish embryo model for TB in combination with a GFP-Lc3 reporter line developed in the Klionsky lab<sup>99,100</sup>. By correlative light electron microscopy studies using the GFP-Lc3 autophagy reporter line we demonstrated the delivery of *Mm* to autophagic compartments<sup>79</sup>. Furthermore, we observed using electron microscopy that double membraned autophagic vesicles fuse with larger *Mm*-containing degrading compartments, a mechanism proposed to enhance the microbicidal capacity<sup>53,62</sup>. We have also shown the protective role of the DNA-damage regulated autophagy modulator *Dram1*, which is upregulated during infection by the central Myd88-NFκB signalling pathway<sup>62,101</sup>. Moreover, using CRISPR/Cas9-mediated mutagenesis, we showed the requirement of selective autophagy receptors Optineurin and p62 for host resistance to mycobacterial infection<sup>52</sup>. For this thesis we took advantage of the possibilities of the zebrafish embryo model for TB and the available zebrafish toolkit to study several autophagy-modulating HDTs as potential anti-TB drugs.

## Outline of this thesis

New drugs for use as TB treatment are needed due to the constraints of classical antibiotics against TB and the rise of antibiotic-resistant strains, making TB a harder

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and harder disease to treat. This thesis is focused on using the *in vivo* whole animal zebrafish embryo model for TB to evaluate potential anti-TB HDTs arising from *in vitro* screens and gain more mechanistic insights into the molecular function of these potential drugs. Although *in vitro* screens for HDTs using cellular models can be performed at high throughput, a limiting step is the validation in whole animal models and translation of results to clinical applications.

The zebrafish model is highly suitable as an intermediate for translational research as it fills the gap between *in vitro* research and mammalian animal models. Research into enhancing the potential of the zebrafish model, such as robotic injection of zebrafish eggs and rapid screening based on automated fluorescence assessment and sorting has led to new developments that make the zebrafish a moderate to high throughput model. In **chapter 2** we used machine learning to improve robotic injection efficiency and effectivity for genetic manipulation of zebrafish larvae using morpholinos, Tol2 transgenesis and the CRISPR/Cas9 system. Robotic injection has similar efficiency as manual injections, but due to its higher throughput leads to a higher yield. This allows for high throughput knock-out or knock-in applications using the zebrafish model.

Due to the complex infection dynamics of mycobacteria, the use of whole animal models is indispensable in research into TB and the zebrafish model has contributed key findings about host-pathogen dynamics during mycobacterial infection. In **chapter 3** we tested several variations of established zebrafish infection protocols to determine which robotic or manual injection conditions are the most suitable to do an initial whole animal screen of potential anti-TB HDTs. We concluded that the manual intravenous injection of *Mm* into one day old embryos gave the most robust results. We then continued with a pilot screen and confirmed the anti-TB activity of Trifluoperazine, Amiodarone and Tamoxifen, first shown in *Mtb*-infected human macrophages, in the zebrafish model for TB.

One of the most promising host targets of HDTs is autophagy. Besides the role of autophagy in cellular homeostasis, the role of autophagy in the immune system has become more and more clear in the last two decades. Intracellular pathogens, such as *Mtb*, are degraded by the autophagy pathway. However, *Mtb* has remarkable strategies to evade degradation and escape from (auto)phagosomes. Therefore, enhancing the autophagic capabilities of professional phagocytes, such as macrophages, is a highly interesting strategy to combat intracellular pathogens and in particular *Mtb*. We used both a primary human macrophage *Mtb* infection model and the zebrafish-*Mm* TB infection model to demonstrate the potential of Tamoxifen as an anti-TB HDT in **chapter 4**. We show the anti-mycobacterial effects are independent of the well-known target of Tamoxifen, the estrogen receptor, and show that Tamoxifen modulates autophagy and in particular the lysosomal pathway. Transcriptome analysis and co-localization studies using fluorescent microscopy show lysosomal activation after treatment with Tamoxifen, as well as increased localization of mycobacteria in lysosomes.

Another potential drug that is interesting as a potential HDT against TB is Amiodarone. This antiarrhythmic medication can induce autophagy and stimulates nitric oxide release. Nitric oxide plays a key role in immunity and inflammation and mycobacteria have been shown to be highly susceptible to reactive nitrogen species. In **chapter 5**, Amiodarone is confirmed to restrict mycobacterial infection in the zebrafish embryo model for TB. We then unravel aspects of host-mechanisms involved in the anti-mycobacterial effect of Amiodarone. We start by investigating the involvement of the nitric oxide host defence

pathway. Furthermore, we use transcriptome analysis and co-localization studies using fluorescent microscopy which point towards alteration by Amiodarone of host pathways related to autophagy and lysosomal function beneficial for the host during mycobacterial infection. Finally, the findings presented in this thesis are put into the perspective of current knowledge in **chapter 6**.

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