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Boosting the host immune system to fight tuberculosis

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Summary and discussion

Tuberculosis (TB) remains a global health problem caused by the intracellular pathogen *Mycobacterium tuberculosis*. TB is treated with classical antibiotics taken daily for up to six months¹. Unfortunately, classical antibiotics are becoming less effective due to the rise of multi-drug resistant (MDR) and extensively-drug resistant (XDR) *Mtb* strains. Making treatment of TB even more difficult is the latent phase of *Mtb* infection, which can persist for many years before leading to active disease. It is estimated that one-third of the global population carries a latent *Mtb* infection¹. Prevention using a vaccine is currently impossible as the available BCG-vaccine offers only partial protection. In the fight against TB, researchers are now also looking at host-directed therapeutics (HDTs) aimed to boost the host-immune system by modulating host-pathways beneficial for the immune response against *Mtb*.

In 2016, the Nobel Prize in Physiology or Medicine was awarded to Yoshinori Ohsumi for his discoveries of mechanisms of autophagy, a term describing an intracellular degradation pathway essential to maintain cellular homeostasis^{2,3}. The term “autophagy” was coined in 1963 by Christian de Duve to describe intracellular vesicles containing cytoplasmic components^{2,3}. These were the early days of the then newly available high resolution transmission electron microscopy techniques, which led to the first observations of autophagic structures in mammalian cells. The concept and principles of autophagy proposed at that time were proven to be accurate in recent years as researchers further unravelled the underlying molecular mechanisms⁴. Furthermore, in the last two decades it has become apparent that autophagy plays a crucial role in health and disease⁵⁻⁷. In addition to its relation to various non-infectious diseases, autophagy proved highly relevant as a host-protective pathway against intracellular pathogens causing some of the most dangerous infectious diseases^{8,9}. The host-protective role of autophagy sparked a particular interest in potential HDTs that modulate autophagy in our endeavour to identify new anti-TB drugs.

Using the zebrafish model to evaluate host-directed therapeutics against tuberculosis

As a starting point for the discovery of HDTs for TB, many studies utilize drug repurposing by screening compounds that have at least passed phase-I clinical trials, and are sometimes approved drugs that are used clinically for other purposes. Most of these high-throughput screens use *in vitro* cell culture, enabling fast identification of potential HDTs¹⁰⁻¹². Positive hits are then moved forward to more complex *in vitro* or *in vivo* models required for validation. To make the translation to the clinic, the use of mammalian models is essential. Zebrafish (*Danio rerio*) are a powerful intermediate model for translational research that fills the gap between *in vitro* research and mammalian models¹³⁻¹⁵. Furthermore, in addition to *in vitro* or *ex vivo* models such as immortalized or primary human macrophages, zebrafish offer a whole animal model that is well accessible for elucidating the molecular mechanisms mediating HDT effects. The whole organism context is of great benefit for TB research, as the interplay between mycobacteria and host cells and tissues during infection is complex, especially due to the role that granulomas (infected cell aggregates) play in TB pathology^{16,17}. Zebrafish are easily genetically manipulated and a wide range of transgenic reporter lines exists that help to identify immune cell types and to analyse immune defence responses¹⁸⁻²⁰. Due to the optical transparency, zebrafish embryos and larvae allow for extensive and detailed live imaging of cellular and intracellular mechanisms. In our study we infected zebrafish embryos with the intracellular pathogen *Mycobacterium marinum* (*Mm*), a close relative of *Mtb*, which is widely used as a model for TB^{14,21,22}.

Zebrafish can be used to perform chemical and genetic screens, aided by robotic injection

techniques and automated fluorescence assessment^{23,24}. Microinjection of zebrafish is a powerful technique to achieve infection with pathogens, introduce cancer cells or compounds, or achieve genetic manipulation by injecting DNA constructs, mRNA, or knock-down/knock-out/knock-in reagents^{14,25,26}. Efficient genetic manipulation requires injection into the cell at the one-cell-stage, and although manual injection is laborious work, this precision could not yet be achieved using robotic injection techniques that were designed to inject in the centre of the yolk. To improve automated injection efficiency for use in genetic manipulation, we used deep learning image recognition (chapter 2). This allowed for recognition of the cell and automated injection close to or in the cell. We achieved similar efficiency levels compared to manual injection and combined with a higher throughput this approach can achieve a higher yield (chapter 2). Deep learning image recognition might eventually also facilitate the development of more complex automated injections into locations desired for infection studies, including the blood circulation and body cavities. However, the robotic technology is not yet sufficiently advanced for these applications. While bacteria can be injected into the yolk using robotic injection, we found the yolk infection approach unsuitable for our study into HDTs (chapter 3). Interaction between the host immune system and the pathogen is needed for HDTs to exert their effect. In the yolk infection model interaction between injected *Mm* and the innate immune system only starts at 2 to 3 dpf when bacteria are able to infect tissues of the developing embryo²³. Immune cells do not migrate into the yolk, which therefore remains a safe reservoir for *Mm*²⁷. In contrast to robotic injection, manual zebrafish injection techniques can be done at multiple timepoints and multiple injection sites, to achieve a variety of goals. For instance, intravenous injection of pathogens into the blood island at 1 day post fertilization (dpf) or at the duct of Cuvier at 2 dpf leads to systemic infection, while localized infection can also be achieved when injection into the hindbrain ventricle or the otic vesicle²⁸.

We compared the blood-island method with the duct of Cuvier method and used fluorescent microscopy to assess bacterial burden and potential developmental toxicity (chapter 3). As we started drug treatment about 1 hour post infection (hpi), developmental toxicity could be minimized using the duct of Cuvier method which is performed at 2 dpf, when the embryo is more developed compared to the blood-island method at 1 dpf. However, as the end-point of the experiment is set at 5 dpf because of animal experimentation regulation, the experimental window for the duct of Cuvier method is 3 days compared to 4 days for the blood island method. While the longer experimental window of the blood island method is an advantage, it is likely that developmental toxicity prevented us from validating HDTs using this system. For instance, when we treated zebrafish embryos with Haloperidol, we observed massive oedema and this phenotype was exacerbated when treatment was performed on infected embryos, rendering Haloperidol unsuitable for experiments in the zebrafish embryo model of TB (chapter 3). However, Haloperidol was shown to reduce intracellular *Mtb* survival in human cells²⁹. In addition, we were unable to confirm several other HDTs that had previously been shown to reduce mycobacterial burden in *in vitro* systems, despite that application of the duct of Cuvier method minimized developmental toxicity. We then reverted to the blood island method to do a pilot screen of potential HDTs identified in an *in vitro* screen. We chose to perform a small pilot screen so that we could test large numbers of larvae to ensure robust effects. However, it is interesting to note that application of the zebrafish embryo model for a large screen for anti-TB compounds recently also proved to be feasible. A screen of 1200 compounds yielded 8 hits of which the most effective was the compound Clemastine, which was found to act as an HDT on the purinergic receptor P2RX7 and to modulate inflammation-associated signalling³⁰. This study shows that by minimizing the number of larvae assessed in the

primary screen it is still possible to find HDTs able to reduce bacterial burden, although this approach will obviously result in many false negatives. In our small pilot screen of 10 compounds, we found 3 compounds to reduce bacterial burden in the zebrafish TB model: Trifluoperazine, from a library of deubiquitinase inhibitors, and Tamoxifen and Amiodarone, from a library of autophagy modulating compounds (chapter 3). We subsequently focused on the two potential autophagy modulators and used the zebrafish embryo model to gain more mechanistic insights into the anti-mycobacterial effect exerted by these drugs (chapters 4 and 5).

Repurposing Tamoxifen as potential host-directed therapeutic for tuberculosis

Tamoxifen is widely known for its use in breast-cancer therapy. The main target of Tamoxifen is the estrogen receptor (ER). Tamoxifen can function as an agonist or antagonist of the ER, which is dependent of the tissue and determined by presence of co-regulatory transcription factors³¹. There is evidence that the inhibitory effect of Tamoxifen on intracellular *Toxoplasma* growth is mediated in a host-directed manner by inducing autophagic degradation of the parasite-containing vacuole¹². Our results show that Tamoxifen inhibits mycobacterial infection in human macrophages and in the zebrafish embryo model of TB (chapter 4). Though several studies found Tamoxifen to have direct antibacterial effects against intracellular pathogens^{32,33}, we found no direct anti-mycobacterial effect of Tamoxifen on *Mtb* or *Mm* at doses that inhibited mycobacterial infection in macrophages or zebrafish, and therefore we propose that Tamoxifen functions as an HDT capable of modulating the immune response against mycobacteria (chapter 4). Tamoxifen was also recently found to have an immunomodulatory effect against MDR gram-negative bacteria³⁴. The therapeutic potential of Tamoxifen is further supported by a recent study that proposed another breast-cancer drug, Bazedoxifene, as an HDT for TB³⁵. Bazedoxifene and Tamoxifen are structurally and functionally related and both target the ER. However, our studies into the host-mediated action of Tamoxifen showed that its anti-mycobacterial effect operates independent of ER signaling and we propose that the HDT effect of Tamoxifen is mediated primarily by enhancing autolysosomal or phagolysosomal degradation pathways.

Both *in vitro* and *in vivo* functional assays and transcriptome profiling revealed major effects of Tamoxifen on autophagy and lysosomal processes (chapter 4). We found an autophagy-increasing effect of Tamoxifen treatment in human macrophages as well as in zebrafish embryos. However, although Cyto-ID staining of autophagic compartments pointed towards an increase in colocalization with *Mtb* in primary human macrophages, we were unable to demonstrate an increase in colocalization of *Mm* with the autophagy marker GFP-Lc3 in zebrafish. It remains possible that an increase of autophagosome formation and maturation contributes to the HDT effect of Tamoxifen. For example, despite our observation that Tamoxifen did not lead to a detectable increase in colocalization of GFP-Lc3 positive vesicles with *Mm*, the observed increase in Cyto-ID and GFP-Lc3 positive vesicles might indicate increased generation of neo-antimicrobial peptides³⁶. Therefore, it is possible that part of the anti-mycobacterial effect of Tamoxifen could be attributed to an increase in the generation and delivery of neo-antimicrobial peptides to mycobacteria-containing compartments, a process in which GFP-Lc3 signal might be rapidly lost due to fusion with lysosomes. This process may work in concert with increased lysosome-mediated degradation of bacteria and be part of an increased killing capacity.

We further analyzed the effect of Tamoxifen by using LysoTracker dye as a fluorescent

staining method for lysosomal acidification. Importantly, we observed not only increased LysoTracker signal but also increased colocalization between mycobacteria and LysoTracker signal in both human macrophages and zebrafish larvae (chapter 4). Although mycobacteria are known to be relatively tolerant to lysosomal acidification and even capable of replication in acidic lysosomes to some extent^{37–39}, in our study Tamoxifen reduced bacterial burden which suggests that the increased numbers of lysosomes and colocalization with mycobacteria is related to an increased killing capacity of macrophages in both human and zebrafish assays. Based on our functional and transcriptomic data on both autophagy and lysosomal modulation we propose that Tamoxifen stimulates *de novo* lysosomal biogenesis and primarily restricts mycobacterial growth by modulation of the (auto)phagosome maturation processes that deliver bacteria to lysosomes (Figure 1A).

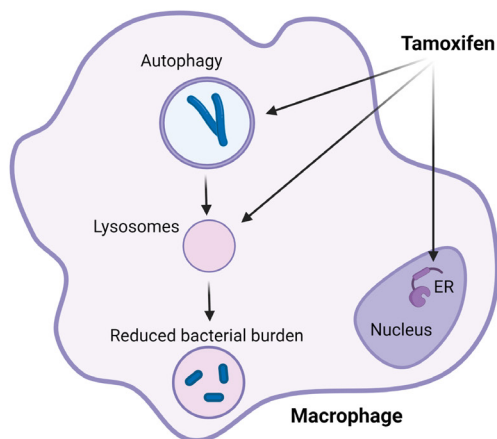
Host-directed therapy with Amiodarone restricts mycobacterial infection and enhances reactive nitrogen levels, autophagy and lysosomal activity

Amiodarone is currently used as an antiarrhythmic drug. It functions by blocking calcium, sodium and potassium channels and inhibiting alpha- and beta-adrenergic receptors. It also causes vasodilation via NO release, which is suggested to aid in the cardiovascular protective properties of Amiodarone^{40,41}. Reactive nitrogen species (RNS) are host protective against pathogenic mycobacteria and are derived from NO^{42,43}. It has been shown that Amiodarone induces autophagy and accumulates in acidic organelles, which suggests it also interacts with other intracellular degradation processes such as the endocytic pathway^{44–46}. We show reduced bacterial burden and an increase in both reactive nitrogen species (RNS) and (auto)phagolysosome activity after Amiodarone treatment in the zebrafish embryo model of TB (chapter 5).

The increase in RNS production by Amiodarone was observed both in neutrophils and in macrophages of zebrafish larvae by measuring the α -nitrotyrosine signal that results from exposure to RNS. In line with previous results⁴⁷, we observed RNS production mostly in neutrophils in both non-infected as infected larvae. Though we also observed increased RNS production in infected macrophages, these levels were far below that found in neutrophils (chapter 5). However, we were unable to demonstrate increased colocalization of *Mm* and α -nitrotyrosine signal and when using inhibitors of RNS production we still observed decreased bacterial burden after Amiodarone treatment (chapter 5). It is important to note that in previous studies using the zebrafish embryo model of TB, RNS defences were activated prior to infection^{47,48}, while we performed Amiodarone treatment post infection. Furthermore, it has been reported that mycobacteria are able to counteract RNS host defences⁴⁸. Taken together, the RNS increase possibly contributes to the HDT effect of Amiodarone, but is unlikely to be the main explanation.

In agreement with autophagy inducing properties reported for Amiodarone, we observed an increase in autophagic vesicles after Amiodarone treatment of zebrafish embryos. However, we were unable to demonstrate increased colocalization of *Mm* clusters and GFP-Lc3 signal. Transcriptome and LysoTracker analysis did reveal involvement of the (auto)phagolysosomal pathway. We found Amiodarone increased LysoTracker positive vesicle numbers and size (chapter 5). These results are in line with results that show Amiodarone restricts viral replication due to accumulation of Amiodarone in endosomes and lysosomes⁴⁵. In this study Ebola and SARS viral particles were contained in endocytic and lysosomal compartments which prevented the release of these particles in the cytoplasm. This effect of Amiodarone has even been suggested to make Amiodarone a potential drug candidate to treat Covid-19⁴⁹. That Amiodarone combines anti-viral and

A



B

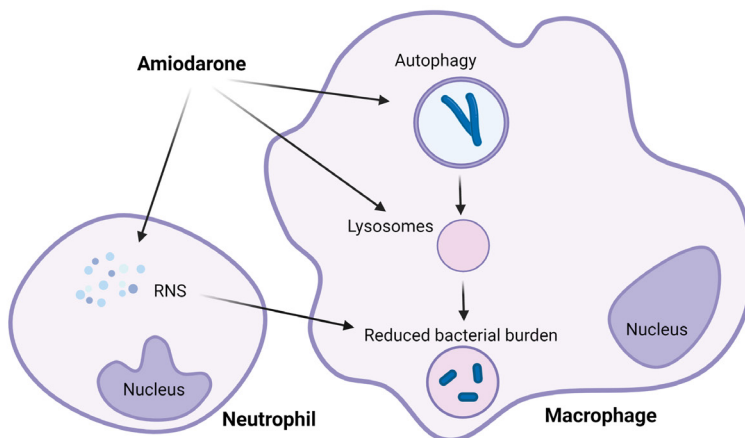


Figure 1. Model of the modes of action of Tamoxifen and Amiodarone

- A. The main target of Tamoxifen is the estrogen receptor (ER). Tamoxifen can also induce autophagy and modulate lysosomal processes. Tamoxifen treatment leads to a reduced bacterial burden, which is independent of ER signalling.
- B. Amiodarone can induce RNS production, autophagy and modulate lysosomal processes. Amiodarone treatment leads to a reduced bacterial burden.

anti-mycobacterial properties is of particular interest considering clinical data pointing to more severe Covid-19 disease consequences for TB patients⁵⁰.

In line with the increased lysosomal staining observed in Amiodarone-treated zebrafish, Amiodarone is known to induce the accumulation of phospholipids in lysosomal structures, called phospholipidosis^{51–53}. Similar phenotypes can be observed in zebrafish models for lysosomal storage disorders³⁸. Increase in intracellular cargo contained in lysosomes can be beneficial either for the host or for the mycobacteria. The key to this balance could be moderation, as severe lysosomal storage defects lead to macrophage necrosis and subsequent exacerbated extracellular bacterial growth³⁸, but

moderate reduction of macrophage migration and increased microbicidal capacity due to increased intracellular cargo contained in lysosomes has a host-protective effect⁵⁴. Possibly, the drug treatment conditions used in our study induced a moderate increase in lysosomal activity and thus tipped the balance towards a host-beneficial effect.

Taken together, we show that Amiodarone modulates two relevant pathways in cellular defence, though we have not fully elucidated the mechanism by which Amiodarone treatment results in lower mycobacterial burden. We demonstrate Amiodarone increases RNS activation and (auto)phagolysosomal pathways and we propose that because of this activation intracellular bacteria are less successful in resisting degradation (Figure 1B). This makes Amiodarone a highly interesting compound to further study as a potential HDT against TB.

Prediction of potential host-directed therapeutics based on our results in the zebrafish model for tuberculosis

In this thesis two HDTs are extensively studied: Tamoxifen and Amiodarone. These compounds share a number of characteristics (chapters 4 and 5)^{12,45}. First, both are known to induce autophagy. Second, both were identified as potential HDTs against *Mtb* in human cells, emerging as hits in a screen of an autophagy modulating compound library. Third, both were confirmed to reduce mycobacterial burden *in vitro* and *in vivo* in a host-directed manner. Fourth, both were found to increase not only autophagy but also the autophagolysosomal axis. However, the targets and molecular function of Tamoxifen and Amiodarone differ greatly, suggesting that the mechanisms of these two HDTs are different as well.

Despite the differences in molecular function, we were intrigued that these two compounds were both able to reduce bacterial burden, most likely via modulation of similar host pathways, particularly (auto)phagolysosomal processes. Therefore, we looked at overlap in effects on the KEGG pathways and GO categories by Gene Set Enrichment Analysis (GSEA) score in zebrafish treated after Tamoxifen and Amiodarone treatment (Figure 2). Interestingly both compounds also have activity against SARS-

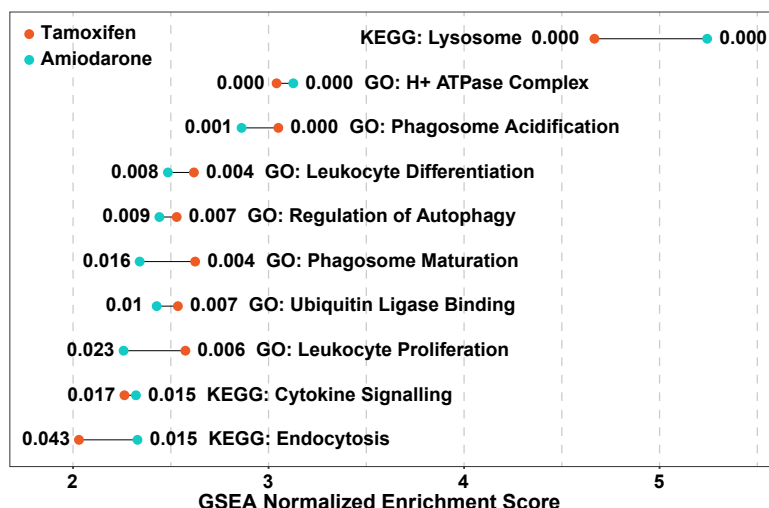


Figure 2. Overlap in the enrichment of KEGG Pathways and Gene Ontology (GO) categories in zebrafish following treatment with Tamoxifen and Amiodarone

CoV-2, which has been attributed to lysosomal effects such as phospholipidosis. To analyse these similarities, we compared the overlapping Tamoxifen/Amiodarone transcriptomic signature to publicly available transcriptomic signatures of drug-studies. This comparison resulted in a list of close to 1800 compounds that show strong effects on pathways related to lysosomal function, and modulate the same genetic pathways as Tamoxifen and Amiodarone. This prediction of potentially effective compounds against mycobacteria includes for instance Dovitinib, which has been shown to be effective against *Mtb* and even MDR-*Mtb*⁵⁵. Interestingly, Dovitinib was identified using a similar *in silico* predictive approach, though this approach was based on drug targets identified in *in vitro* human cell cultures. The list also includes the compound YM-201636, which inhibits PIKfyve kinase, recently shown to be involved in Dram1-dependent vesicle delivery to lysosomes⁵⁶. These examples show the potential of using the transcriptomic data obtained after Tamoxifen and Amiodarone treatment in the zebrafish embryo model of TB to predict potential HDTs of interest as anti-TB drugs.

Conclusion

New therapeutic approaches for TB are needed and HDTs can function as adjunctive drugs to first-line antibiotics to shorten treatment time and combat MDR- and XDR-*Mtb*. Screening for potential HDTs can be done rapidly *in vitro*, but in this thesis we show that the zebrafish embryo model for TB can combine screening and validation with mechanistic analysis. The improvements on robotic injection of zebrafish eggs using deep learning described in **chapter 2** offer an insight into the possibilities for future steps in automation of zebrafish research, especially for large-scale screens. The methods using zebrafish evaluated in **chapter 3** provide an overview of approaches that can be used for drug-screens. We highlight potential pitfalls for future drug-screens using zebrafish and validate three HDTs in a pilot screen using our chosen approach of the blood island injection method. We subsequently analysed the underlying mechanisms of the two autophagy-modulating HDTs Tamoxifen and Amiodarone in **chapters 4 and 5**. This thesis shows that both these drugs, which have been used in the clinic for years, can potentially be repurposed for TB treatment due to their stimulatory effects on autolysosomal or phagolysosomal degradation pathways that are important to control infection. Our studies also revealed that these drugs have broad effects on the transcriptome, which could be host beneficial via various mechanisms when used for infectious diseases. These findings underscore the importance of investigating the underlying mechanisms of action of drugs identified in chemical screens. Although further research in mammalian models is necessary to translate the results on Tamoxifen and Amiodarone to the clinic, this thesis shows the relevance of using zebrafish larvae as an intermediate translational vertebrate model.

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