Host-directed therapy for intracellular bacterial Infections

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6 | Summary and Discussion

As outlined in **Chapter 1**, there are many challenges that accompany the relatively new field of research on host-directed therapies (HDT) in infectious diseases, including technical limitations and obstacles. This particularly holds true for the study of virulent pathogens such as *Mycobacterium tuberculosis* (*Mtb)*, and requires the development of novel technologies and approaches. Against this background we set out to develop novel models and tools visualize and quantify bacterial infection in human cellular models, as described in detail in **Chapters 2** and **3**.

Development of a novel flow cytometry-based screening assay for intracellular bacterial infections in human cells

There is a clear need for high-throughput analyses of interactions at the host-pathogen interface as well as the rapid identification of new candidate molecules, including drugs that target the host molecules regulating these interactions. To address these issues, several reports describing high-throughput screens for HDT discovery have been published in recent years. These studies employed a range of different assays, including traditional colony forming unit (CFU) assays[1,2], bioluminescent assays[3,4] and automated microscopy[5,6] to enumerate bacterial loads. CFU assays have been the golden standard in TB research for over a century, but these are not ideal for high-throughput screening as *Mtb* proliferates very slowly, resulting in a 2-3 week minimum waiting period between bacterial plating and colony count readout, which can extend even to 4-6 weeks. Moreover, performing the assay is very laborious, limiting the throughput. Furthermore, on a more technical note *Mtb* colonies have irregular morphologies and sizes, making it hard to distinguish and quantify them accurately. Employing bioluminescence has been proposed as an alternative[3,4], but even though bioluminescent assays are well quantifiable, reliable and rapid, these assays offer only a single-parameter readout and cannot provide information about exact bacterial loads per cell or the fraction of infected cells. Bridging this gap, several studies have used phenotypic screens employing automated microscopy[5,6]. Using these assays, many different parameters can be derived from images, resulting in large quantities of data. This is both an advantage and a drawback of this methodology, as costly storage facilities, computational infrastructure and computational time needed for analysing such large quantities of data in a high-throughput setting are limited in most laboratories. Moreover this technique is often dependent on automated microscopy platforms and proprietary image analysis software that is not widely available. Therefore, we set out to develop a novel flow cytometry-based screening assay that allows quantification of bacterial loads, infected cell populations and host cell viability, employing novel fluorescent reporter strains of *Mtb* and *Salmonella enterica* serovar Typhimurium (*Stm*). The development of this assay is discussed in **Chapter 2**, in which we report the successful application of the assay in chemical compound screens, using up to 1,200 compounds, and in

siRNA screens testing up to 1,000 siRNA pools (used in **Chapters 3** and **4**). Besides the visualization of infected cells, the quantification of bacterial loads per cell and the exclusion of dead cells, another important advantage of this assay is the strongly decreased time between initiation of the experiment and the readout, which is 24-72 hours instead of 2-4 weeks for classical *Mtb* CFU assays.

The first step towards developing this fluorescence-based assay was engineering novel *Mtb* fluorescent reporter strains expressing stable and destabilized DsRed fluorophore variants, respectively. As *Mtb* is a slowly proliferating bacterium, high stability of fluorescent reporters could potentially cause problems, as accumulation of fluorescent proteins can yield a false positive signal from dead bacteria. Although the stability of the fluorophore did not seem to affect the results obtained in our chemical compound treatment experiments in *Mtb*-infected cells, a more sensitive system was required when performing siRNA experiments, as siRNA-mediated genetic silencing typically resulted in more subtle phenotypes than following chemical compound treatment. To this end we developed a destabilized DsRed reporter construct, which decreases the half-life of DsRed from 4.6 days to several hours[7,8], for expression in *Mtb* and showed that this enabled siRNA screening with an excellent signal-to-noise window.

Since they are the natural target cells for *Mtb* infection, human primary macrophages (Mϕs) represent the ideal model system for in *vitro* TB studies. However, in high-throughput settings the use of these cells suffers from several major drawbacks, a major one of which is that isolating sufficient quantities of human primary Mϕs from single donors is not possible and donor-dependent variation cannot easily be controlled for. An alternative often used by researchers is the human monocytic cell line THP-1. However, differentiation and maturation of this cell line towards a Mϕ-like phenotype requires phorbol 12-myristate 13-acetate (PMA) stimulation, which induces significant Protein Kinase C (PKC) activation[9,10]. This altered signaling background is not desirable for HDT studies, as many host-pathogen interactions take place at the cell signaling level. Therefore we sought a suitable human cell line that is able to phagocytose *Mtb* without the need for chemically induced differentiation and which could be validated in our experimental setting (that is: in which HDT results from literature could be faithfully replicated). As we have previously shown that melanocytes possess phagocytic capability[11], we used and validated the human melanoma cell line MelJuSo as a novel *Mtb* infection model in **Chapter 2**. This model has several important advantages compared to the Mϕ and THP-1 models described above. Firstly, MelJuSo is an established cell line, and is more homogeneous than primary cells from different donors. This enabled both upscaling and greater reproducibility. Secondly, in contrast to the THP-1 model, MelJuSo cells do not require additional stimulation to induce a phagocytic phenotype, thereby providing a 'clean' signaling background for chemical and siRNA screens. Thirdly, as we have shown in **Chapter 2**, MelJuSo cells are efficiently transfectable and near-complete knockdown of specific target gene expression can be achieved using siRNA, while siRNA transfection of THP-1 cells was heterogeneous and resulted in incomplete gene knockdown in our experiments. The high transfectability of MelJuSo cells also offers opportunities for studying *Mtb* infection in cells overexpressing genes or expressing (fluorescently) tagged proteins. More

importantly, we show in **Chapter 2** that results obtained using HDT compounds reported in literature can be faithfully reproduced in MelJuSo cells. Of further importance with regard to validating the MelJuSo model, we demonstrated in **Chapters 3** and **4** that our chemical compound screening results obtained in the MelJuSo-*Mtb* model could be validated in a primary Mϕ *Mtb* infection model. Taken together, these findings firmly establish MelJuSo as a suitable and highly versatile novel *Mtb* infection model for chemical genetics studies, including TB HDT studies.

Beyond wet-lab screens: employing *in silico* prediction tools to accelerate HDT drug discovery for TB

In **Chapter 3** we introduce an *in silico* prediction model as a novel approach to accelerate drug discovery, allowing us to identify novel candidate HDT compounds from a virtually unlimited library of compounds (the PubChem repository) by inferring compound target profiles from compound library screening data. As chemical compounds rarely have a single target and their efficacy in a HDT setting may depend on simultaneously targeting multiple host molecules, our prediction tool was geared towards the ranking of target profiles rather than single target species and on subsequently identifying those compounds in the PubChem repository that have similar target profiles to compounds that were efficacious in our compound library screen. This allowed identification of novel active compounds not just by structural similarity, but rather by confirmed target profiles, allowing a search for compounds that have the desired target profile with reduced toxicity. Moreover, by further focusing our search on active target profiles, the prediction model allowed us to identify host molecules and combinations of these molecules that are essential for bacterial survival. Importantly, we identified novel candidate drugs for HDT against *Mtb* (Dovitinib, AT9283 and ENMD-2076) as well as *Stm* (Nafoxidine and Opipramol), which were shown to be efficacious in our (drug-sensitive (DS) as well as MDR-)*Mtb* and *Stm* infection models, including in human primary Mϕs (discussed below). Moreover, as the prediction model was agnostic to our manual hit compound selection, it allowed fully unbiased validation and follow up of the primary compound screen.

As the prediction model used simple numerical values (in our case z-scores) as input, it can in theory be easily adapted to any screen of chemical compounds (provided that the compounds are submitted to the PubChem repository) and may therefore be of interest for research on other druggable diseases, in particular for drug repurposing studies.

Novel targets and drug candidates for HDT

Applying the novel tools we developed above as well as using a zebrafish TB infection model in several individual studies, we identified multiple novel targets for HDT. These include receptor tyrosine kinases (RTKs), PCTAIRE kinases and the DNA-damage regulated autophagy modulator (DRAM1) as new molecular targets for HDT, as well as chemical inhibitors for several of these molecules as starting points for novel anti-infective therapies. These findings will be discussed below, contextualized by other results and published literature.

Role of receptor tyrosine kinases and their chemical inhibitors in mycobacterial infection

In our screen of a Library Of Pharmacologically Active Compounds (LOPAC) in the MelJuSo-*Mtb* model (discussed in **Chapter 3**), 4 out of 5 validated best hit compounds (which inhibited intracellular *Mtb*) were molecules affecting kinases that participate in RTK signaling pathways. These compounds included: Tyrphostin AG 494 (an EGFR inhibitor), SU 6656 (a SRC Family Kinase (SFK) inhibitor), SB 216763 (a GSK-3 inhibitor) and GW5074 (a RAF1 inhibitor) [12-16]. As an independent validation of these findings, our *in silico* predictive model identified additional RTK inhibitors AT9283, ENMD-2076 and Dovitinib as candidate hits, and their activity was subsequently confirmed in our MelJuSo and human primary MΦ (DS and MDR) *Mtb* infection models. Importantly, an unbiased siRNA screen of the human kinome in the MelJuSo-*Mtb* model again independently identified RTK signaling as a host regulatory pathway of *Mtb* infection, and BLK, ABL1 and NTRK1 were identified as candidate targets for HDT. As RTK inhibitors are already an active topic of studies in cancer research[17] and compounds like AT9283, ENMD-2076 and Dovitinib have already entered clinical trials up to phase III[18-23] (http://www.clinicaltrials.gov), RTK inhibitors are promising candidates for drug repurposing and rapid development into HDT drugs for intracellular bacterial infections.

The relevance of (growth factor) RTK signaling pathways for mycobacterial infection is supported by multiple independent studies. Firstly, the growth factor VEGF was shown to be responsible for enhanced angiogenesis in TB granulomas and thereby to support mycobacterial survival in zebrafish *Mycobacterium marinum* (*Mm*) [24] and rabbit *Mtb*[25] infection models. In the study by Oehlers *et al*., Pazopanib (one of the compounds identified by our predictive model) was used to inhibit the VEGF receptor (VEGFR), and this limited granuloma vascularization while decreasing the bacterial burden in their zebrafish *Mm* infection model. Our study showed that VEGFR inhibition may also result in (yet unidentified) intracellular events leading to inhibition of mycobacterial growth, as angiogenesis could not be a contributing factor in our cellular infection model. Secondly, in a chemical screen Stanley *et al*. identified Gefitinib as a compound that inhibits *Mtb* growth by targeting epidermal growth factor receptor (EGFR) signaling. Inhibitors of both these pathways were also identified as host-directed inhibitors of *Mtb*, first in our LOPAC screen (the EGFR inhibitor Tyrphostin AG 494) and subsequently *in silico* in our predictive model (the VEGFR inhibitors Dovitinib and Pazopanib).

Chemical optimization of the H-89 inhibitor scaffold identifies 97i as a novel HDT drug candidate and reveals a role for PCTAIRE kinases in regulation of intracellular bacterial infection

The study by Kuijl *et al*. laid the groundwork for the research reported in this thesis and introduced AKT1 as a promising target for HDT for intracellular bacterial infections[26]. Currently, the role of AKT1 in *Stm* infection remains undisputed. However, the work reported in this thesis sheds new light on its role in regulating *Mtb* infection. Our results reported in **Chapters 3** and **4** confirmed the involvement of AKT1 in *Mtb* infection but also indicated that other host molecules (as discussed above) may be highly significant additional determinants of *Mtb* infection outcome. In contrast to AKT1 as a strong host regulator of *Stm* infection, a single host 'master regulator' of *Mtb* might not exist or has not yet been identified. Our results in **Chapter 4** indicated that indeed a combination of host molecules must be perturbed in order to successfully control intracellular *Mtb*. Our experiments with *Stm* suggested that supplementation of H-89 treatment with silencing of CDK18 could further enhance control of *Stm* bacteria, but this remains currently unproven for *Mtb*. A possible explanation for the requirement for targeting multiple host molecules might be functional redundancy of these targets. However, another possibility might be that *Mtb* employs multiple (simultaneous or consecutive) strategies for evading host control, which should be targeted simultaneously (or consecutively) by novel therapeutics. Further research should therefore focus on identifying the minimal core host molecules that control *Mtb* infection and identifying or developing specific compounds targeting these.

Directly building upon the study by Kuijl et al. [26], we used the kinase inhibitor H-89 as a starting point for further development of host-directed compounds that inhibit intracellular bacterial infections (**Chapter 4**). In **Chapters 3** and **4** we demonstrated that host-mediated bacterial inhibition was significantly stronger in *Stm*-infected cells than in cells infected with *Mtb,* sparking our efforts to enhance the target specificities of the H-89 scaffold in search of the minimal core host molecules that control *Mtb*. This study represents an example of how lead compound optimization can result in significantly enhanced bacterial inhibition by optimizing target specificities. By altering the chemical structure of H-89, we were able to generate host-directed compounds with significantly enhanced activity against intracellular *Mtb*. From this library, compound 97i was identified as the lead molecule with the greatest potential for drug development. Using kinome profiling, we were able to identify the PCTAIRE kinases, a cyclin-dependent kinase (CDK) subfamily consisting of CDK16, CDK17 and CDK18 (PCTAIRE-1, PCTAIRE-2 and PCTAIRE-3, respectively), as host molecules that were targeted by 97i. While H-89 inhibits CDK16 and CDK17, we demonstrated that 97i targets all three PCTAIRE kinases. When CDK18 was genetically silenced in combination with H-89 treatment, inhibition of the outgrowth of intracellular *Stm* was indeed improved, suggesting that all three PCTAIRE kinases must be inhibited for optimal inhibition of bacterial growth, possibly due to functional redundancy of the members of this kinase family. Follow-up studies will need to extend this to *Mtb*.

The PCTAIRE kinases are cytosolic kinases that up until now have been poorly characterized[27]. Their contribution to cell cycle regulation (a hallmark of many CDKs) is disputed and their cellular-molecular functions are largely unknown. However, several recent studies have provided possible avenues for further research to elucidate their role in controlling intracellular bacterial infections. Firstly, different PCTAIRE kinases have previously been linked to intracellular vesicle transport. In a study by Palmer *et al*., direct interactions of the PCTAIRE kinases CDK16 and CDK18 with COPII coatomer proteins, which regulated vesicle transport from the endoplasmic reticulum (ER) were demonstrated and this process could be partially blocked by H-89 treatment[28]. COPII-coated vesicles are involved in anterograde transport of protein products from the ER tot the Golgi apparatus and can therefore directly impact delivery of proteins to the phagosome or other vesicles[29]. The notion that inhibition of PCTAIRE kinases blocks both ER export as well as *Stm* (and possibly *Mtb*) outgrowth suggests that these intracellular bacteria might either require functional protein transport in the host cell for their survival or that the bacteria 'hijack' cellular processes involved in vesicle transport. As we showed in **Chapter 4** that 97i inhibits all three PCTAIRE kinases, the study by Palmer *et al*. might thus provide a basis for elucidating the mechanism responsible for bacterial inhibition by the host. A role for PCTAIRE kinases in vesicle transport is further supported by a study from Matsuda *et al*. [30]. The authors demonstrated that CDK18 regulates the RhoGTPases RAC1 and RHOA through inhibition of the kinase FAK, indicating that CDK18 might be indirectly involved in regulation of cell motility and adhesion, phagocytosis and phagosome maturation through reorganization of the actin cytoskeleton[31,32]. We previously also identified a role for RAC1 and RHOA during *Stm* infection and AKT1 was shown to be a regulator of this process by targeting PAK4 and thereby modulating GEF-H1 activity. The CDK18-FAK axis might present an alternative route to modulate RAC1 and RHOA activity during bacterial infection[26]. Our own preliminary siRNA screening data also support a role for FAK in controlling intracellular bacteria, as silencing of this kinase promoted outgrowth of both *Stm* and *Mtb*. Secondly, PCTAIRE kinases might be involved in immune modulation, which could impact bacterial control. CDK16 knockdown was recently shown to enhance sensitivity of tumor cells to TNF-family cytokines[33], and TNF is a major protection-associated cytokine in TB[34]. Whether inhibition of PCTAIRE kinases (for instance by 97i) might have an additional beneficial effect through sensitization of infected Mϕs to TNF-family cytokines remains to be studied in more complex models than the cell lines and Mϕs that we employed in our studies. So far, our own preliminary data indicated that 97i might shift *Mtb*-infected Mϕs towards a pro-inflammatory Mϕ1-like phenotype, as we observed decreased cell-surface expression of the Mϕ2 marker CD163 and a shift towards pro-inflammatory cytokine secretion upon treatment with 97i. Another possible immunomodulatory role for PCTAIRE kinases, namely at the level of the IL-1β-induced inflammatory response, was reported by Frank *et al*. [35]. In their study, the authors identified CDK18 as one of the proteins required for inhibition of the IL-1β-induced inflammatory response by *Klebsiella pneumoniae* (*K. pneumoniae*). As an important role for IL-1β in skewing the type I interferon response towards a protective phenotype during *Mtb* infection was recently demonstrated[36], this may

suggest another possible mechanism for PCTAIRE kinase-mediated control of intracellular bacteria. Interestingly, inhibition of the IL-1β-induced inflammatory response by *K. pneumoniae* was shown to be EGFR-dependent by Frank *et al.*, providing an interesting link between RTK inhibitors (discussed above and in **Chapter 3**) and PCTAIRE kinase-mediated modulation of IL-1β-induced inflammation.

DRAM1

In another series of studies that focused on the zebrafish-*Mm* infection model and on *Mtb* infection of human Mϕs, we described the identification of DNA Damage-Regulated Autophagy Modulator (DRAM1) as a new molecule in host resistance against mycobacteria, and thus also as a new candidate target for HDT (**Chapter 5**). DRAM1 was previously known as a protein that induces autophagic and cell death responses following cancer-related cellular stress or HIV infection, both in a p53-dependent manner. In the embryonic zebrafish-*Mm* infection model, however, we observed that Dram1 is also regulated by the TLR/IL1R-MYD88-NF-κB axis, in a manner independent from p53. Importantly, we demonstrated that *DRAM1*/*dram1* is upregulated in response to mycobacterial infection in zebrafish (*Mm*) as well as in human primary Mϕs (*Mtb*) and that DRAM1/Dram1 colocalizes with intracellular mycobacteria in both models. As *dram1*-deficient zebrafish embryos were incapable of containing mycobacteria in vesicles, *Mm* infections resulted in excessive bacterial loads in these embryos, indicating a direct involvement of Dram1 in mycobacterial control. We further demonstrated that TLR/IL1R-MYD88-NF-κB-dependent up-regulation of Dram1 was a prerequisite for targeting the autophagic response to mycobacteria and that this response required p62 as well as the DNA-sensing Sting pathway. In human Mϕs the STING pathway was previously shown to induce ubiquitination of *Mtb* in phagosomes that had been damaged by a *Mtb*-RD1 locus encoded virulence factor[37]. Romagnoli *et al*. showed in *Mtb*-infected dendritic cells that RD1-related virulence may be responsible for inhibition of autophagy and promotion of mycobacterial survival, suggesting that this host-pathogen interaction may be exploited to enhance autophagic control of mycobacterial infection[38]. Our results corroborate this notion, as indeed Sting was essential for induction of selective autophagy during mycobacterial infection. Therefore, targeting the TLR-MYD88-NF-κB axis to activate DRAM1 or downstream effectors is a potential strategy for HDT to overcome inhibition of selective autophagy by mycobacteria.

Additional compounds and targets

Our chemical and siRNA screens in **Chapters 3** and **4** identified additional compounds and host molecules that could not actively be pursued further in our studies but that might be of interest for future HDT research.

In our LOPAC screen, also host-directed compounds other than RTK signaling inhibitors were identified that affected intracellular *Mtb* growth. These compounds target a range of different host molecules (**Chapter 3**). One of these compounds was Quinacrine, an antimalarial drug with reported activity in various other diseases through several different targets[39]. Of note, AKT1 (discussed in detail in **Chapter 1** and below), NF-κB (a possible link with DRAM1, which is

described in **Chapter 5** and discussed above) and phospholipase A2 (a key enzyme in the eicosanoid pathway, previously identified as a regulator of the type I interferon response in *Mtb* control[36]) have been reported as targets of Quinacrine[40]. Furthermore, compounds targeting Ca^{2+} transport were identified in our screens in the MelJuSo-*Mtb* model (3',4'-Dichlorobenzamil[41]) and HeLa-*Stm* model (Mibefradil[42]). As Ca^{2+}-mediated activation of calcineurin was previously proposed as a mechanism inhibiting phagosome maturation in *Mtb*-infected cells[43], modulating intracellular Ca^{2+} transport using 3',4'-Dichlorobenzamil or Mibefradil might promote phagosome maturation and control of intracellular bacteria in infected cells.

Additionally, our screens independently confirmed the significant activity of the previously reported candidate HDT compound Haloperidol[5] and the role of the human kinase ABL1[44,45], further supporting the plausibility of our model system as discussed above. Haloperidol, an antipsychotic that targets dopamine receptors[46], was previously shown to inhibit mycobacterial survival by accelerating endolysosomal trafficking in human cells[5]. We confirmed this in our LOPAC screen in the MelJuSo-*Mtb* model (**Chapter 3**) and extended this finding by showing that Haloperidol also inhibited *Stm*, indicating that this compound may offer a wider range of HDT applications. Interestingly, we also identified the Sigma receptor agonist Opipramol as a host-directed inhibitor of *Stm* using the *in silico* predictions based on the LOPAC screening data and subsequently confirmed the host-mediated anti-bacterial activity of this compound *in vitro*. As Haloperidol has been reported as a high affinity Sigma receptor interactor[47], this finding provides further support for Haloperidol as a promising HDT compound and identifies Sigma receptors as potential host targets that may be exploited for HDT. The siRNA screen of the human kinome reported in **Chapter 3** also independently confirmed a role for the non-receptor tyrosine kinase ABL1. ABL1 was previously reported as a host regulator of mycobacterial infection and its inhibitor Imatinib was efficacious in inhibiting *Mtb in vivo*[44,45]. Importantly, we confirmed the activity of Imatinib in our MelJuSo-*Mtb* model in **Chapter 2**, but Imatinib did not surpass the level of *Mtb* inhibition exerted by H-89 in our model. A possible explanation for this might be that Imatinib, in addition to affecting ABL1 signaling, also modulates the myeloid compartment, which may reduce the bacterial burden[48]. As this cannot be measured in a cellular infection model, the effects of Imatinib on *Mtb* infection were likely limited to ABL1-mediated mechanisms in our model, explaining the discrepancy in efficacy with *in vivo* models.

A future perspective on HDT for intracellular bacterial infections

Apart from the novel compounds and host molecules identified for HDT reported above, our studies also provide important insights for additional HDT strategies and the further identification and development of HDT molecules.

In our studies, we used a chemical compound-centric approach followed by target identification, for several reasons. Firstly, as we demonstrated that

perturbation of multiple host molecules may be required for host control of intracellular *Mtb* (discussed in the previous paragraph) and chemical compounds rarely have a single target, efficacious chemical compounds provide an ideal starting point for elucidation of host-pathogen interactions that regulate intracellular bacterial survival. Conversely, in gene silencing approaches individual genes are targeted. In our own experiments, the effect of knockdown of single genes on *Mtb* bacterial loads was limited and relatively few targets were identified that significantly affected *Mtb* survival. However, our siRNA screen of the human kinome confirmed HDT targets reported in literature like ABL1 (discussed above) and provided a significant and valuable complementary approach to chemical compound screening, particularly when studying the dataset using network or pathway analyses rather than by focusing on individual targets, as we demonstrated in **Chapter 3**. Secondly, screening for efficacious compounds instantly provides starting points for drug development, whereas in genetic approaches compounds targeting the identified gene products must first be found before further development into clinically applicable drugs is possible, if at all. Thirdly, performing chemical screens of existing drugs for drug repurposing may further accelerate HDT drug development as generally a wealth of drug safety and pharmacokinetic data is available and the drugs may either be in, or already have passed the stage of clinical trials, thus enhancing the chances of successful clinical application. This is exemplified by our LOPAC screen and the subsequent *in silico* predictions in **Chapter 3**, where we identified multiple compounds as HDT candidates that were already studied in clinical trials.

Even though the focus on chemical compounds described above is the fastest way towards clinically applicable HDT drugs, fundamental research (and systems biology approaches) will remain essential to expand our understanding of the host-pathogen interactions that underlie the successful survival of major pathogens like *Mtb* inside host cell niches. By definition, pathogens are organisms that have acquired some level of resistance against innate host microbicidal mechanisms as this is a prerequisite for successful colonization. Even though HDT approaches are less likely to cause resistance than direct anti-microbials (as discussed in **Chapter 1**), because the former do not act on bacterial targets that can be rapidly mutated, but rather on host targets that cannot, selective multi-pronged host-mediated immune pressure is still exerted on the bacteria. Therefore, at least in theory, it cannot be excluded that chemical perturbation of host mechanisms may lead to the emergence of pathogens that have developed alternative methods for escaping host immune functions. A thorough understanding of the interactions taking place at the host-pathogen interface and downstream pathways and effector mechanisms is therefore essential to prevent development of novel 'HDT-resistant' pathogens.

By performing our studies in both *Stm* and *Mtb* infection models we gained important insights in the applicability of HDT for different pathogens. As a basis for our studies, we showed in **Chapters 2** and **4** that H-89 is not equally effective in *Stm* and *Mtb* infection models. In addition, the chemical compound screens described in **Chapters 3** and **4** showed limited agreement between the *Stm* and *Mtb* models. This indicated that future HDT approaches must take specific perturbations of host immune mechanisms by different pathogens into

account, again underscoring the need for fundamental understanding of host-pathogen interactions. Despite this, we identified several compounds that displayed activity in both the *Stm* and *Mtb* infection models. Most notably, H-89-derived kinase inhibitor 97i (**Chapter 4**) was highly potent in inhibiting both *Stm* as well as *Mtb* in our experiments and we showed in **Chapter 3** that Haloperidol (which was previously shown to inhibit mycobacterial infection) also inhibits intracellular *Stm*, providing important proof-of-principle for wide-spectrum HDT. Therefore, identifying the strategies for bacterial survival that are employed by multiple pathogens may identify critical host molecules or pathways that can be exploited for wide-spectrum HDT.

In conclusion, HDT presents a promising novel strategy in the fight against global antibiotic resistance. In this newly emerging field, significant efforts will be required to develop clinically applicable HDT drugs. Detailed knowledge of the interactions taking place at the host-pathogen interface should be expanded to identify the most promising avenues for HDT against rampant anti-microbial resistant infections such as MDR, XDR and TDR TB and typhoid disease. The work described in this thesis contributes to this through the identification of multiple new targets and chemical compounds that can help accelerate development of novel HDT drugs, either directly by providing repurposable drugs with established clinical applicability, as well as by identifying novel host molecules and pathways contributing to the fundamental understanding of host-pathogen interactions in TB and typhoid disease.

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