Identification and characterization of novel antitubercular compounds

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Abstract

Novel strategies, including phenotypic screenings directly in a host-pathogen system, are needed to discover new drugs against tuberculosis. We used amoebae such as Dictyostelium discoideum and Acanthamoeba castellanii as a host model for in vivo drug screenings. We developed assays to monitor the impact of chemicals and intracellular factors on bacteria health, growth, and virulence mechanisms and intrinsic host defenses. We used GFP fluorescent reporters for quantification of bacteria numbers. We validated the system using known first and second line antimycobacterial drugs.
Identification and Characterization of Novel Antitubercular Compounds

THÈSE
présentée à la Faculté des sciences de l’Université de Genève
pour obtenir le grade de Docteur ès sciences, mention biochimie

par

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de

Novosibirsk (Russie)

Thèse N° 4775

GENÈVE
Repromail
2015
Doctorat ès sciences
Mention biochimie

Thèse de Monsieur Valentin TROFIMOV

intitulée :

"Identification and Characterization of Novel Antitubercular Compounds"

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0.1 Résumé

Plus d’un tiers de la population mondiale est infectée par *Mycobacterium tuberculosis*, avec pour conséquence 2 millions de morts et 8 millions de nouveaux cas d’infection chaque année. De plus, l’efficacité des thérapies existantes est menacée par une augmentation dramatique du nombre de souches multi-résistantes. De nombreuses drogues potentiellement anti-tuberculeuses identifiées par des criblages in vitro sur *M. tuberculosis*, n’ont pas prouvée leur efficacité dans des systèmes in vivo. Il est donc nécessaire d’établir de nouvelles stratégies afin de découvrir des composés ayant un haut potentiel d’activité anti-bactérienne in vivo, comme des criblages phénotypiques directement chez le système hôte-pathogène.

Les amibes, comme *Dictyostelium discoideum* et *Acanthamoeba castellanii*, permettent d’effectuer des criblages de drogues de moyen à haut rendement. Nous avons élabordé des méthodes permettant de mesurer l’impact de composés chimiques et de facteurs intra-cellulaires sur la santé, la croissance et les mécanismes de virulence de souches bactériennes ainsi que sur les défenses intrinsèques des hôtes. Nous avons utilisé des souches de *M. marinum*, *Dictyostelium* et *Acanthamoeba* exprimant des rapporteurs fluorescents tels que mCherry ou GFP. Nous avons validé notre système en utilisant des drogues anti-mycobactériennes connues de première et deuxième ligne, telles que l’isoniazide, la rifampicine, la pyrazinamide, l’ethambutol, la streptomycine, l’amikacine et la kanamycine. Les mesures de fluorescence obtenues ont été confirmées par observation microscopique. Des méthodes de microscopie “hight-content” ont également été développées afin de valider les résultats dans une lignée cellulaire mammifère.

Nous avons tout d’abord criblé une banque de 1224 composés élaborés par le groupe du docteur Scapozza (Université de Genève). Ce crible a permis d’identifier des composés ayant une activité anti-infectieuse mais également des composés ayant une activité pro-infectieuse. 1.7% des composés de cette banque ont démontré l’une ou l’autre de ces activités. Les composés indentifiés ont été validés et des courbes “doses-réponse” ont été générées. L’activité de ces composés a été validée en mesurant leur impact sur l’infection de cellules *Dictyostelium* et de cellules microgliales mammifères à l’aide de microscopie “high-content”.

Nous avons également commencé à étudier la relation structure-activité de ces composés. D’autres banques de composés telles que GlaxoSmithKline TB set, la Malaria box et la Prokinase library ont également été criblées.
0.2 Summary

Over one third of the world population is infected by *Mycobacterium tuberculosis*, resulting in 2 million deaths and 8 million newly infected people every year. In addition, the efficiency of the existing therapies is threatened by the dramatic increase of multi-drug resistant strains. Many drug candidates identified by *in vitro* screens on *M. tuberculosis* fail when tested *in vivo* systems. Therefore, novel strategies, including phenotypic screenings directly in a host-pathogen system, are needed to discover antibacterial activities with high *in vivo* potency.

Amoebae such as *Dictyostelium discoideum* and *Acanthamoeba castellanii* allow performing convenient medium to high-throughput drug screenings. We developed assays to monitor the impact of chemicals and intracellular factors on bacteria health, growth, and virulence mechanisms as well as on intrinsic host defenses. We used mCherry and GFP fluorescent reporters for *M. marinum*, *D. discoideum* and *A. castellanii*, respectively. We validated the system using known first and second line antimycobacterial drugs, including isoniazid, rifampicin pyrazinamide, ethambutol, streptomycin, amikacin, kanamycin, confirming the fluorescence measurements by visual inspection. High-content microscopy assays were developed for subsequent validation in a mammalian cell line system.

We first performed a medium-throughput screening with 1224 compounds from a library designed by the group of Dr. Scapozza (University of Geneva), with a final hit rate of about 1.7%. Both anti-infective and pro-infective activities were identified. Identified compounds were validated and dose-dependent curves were generated. The hits were validated in infected *D. discoideum* and mammalian microglial cells by high content microscopy. We have started to investigate structure-activity relationships (SAR) and have also screened additional chemical libraries, such as the GlaxoSmithKline TB set, the Malaria box and the Prokinase library.
1 Introduction
1.1 TB as a health threat

Tuberculosis (TB) is one of the most serious health threats worldwide. This disease is the second leading cause of death from infectious diseases after HIV infection. It is estimated that about 1/3 of the world population is infected. In 2011 8.7 million new cases were registered, and 1.4 million deaths occurred. Among these deaths 990,000 were HIV-negative while 430,000 deaths were HIV-associated (WHO report 2013). Importantly, TB is one of the top killers of women worldwide with 0.5 million cases per 1.4 million TB-related deaths in 2011.

Tuberculosis is strongly associated with developing world due to overall weakening of immune system largely caused by inadequate hygienic conditions as well as high rates of HIV infection and the corresponding development of AIDS (Lawn and Zumla 2011). The highest rates of TB infection are in Asia and Africa (Fig.1.1). Overall China and India together provide 40% of the registered cases of TB while Africa has one quarter of the cases and has the highest mortality rate. Notably, 3.7% of the new cases worldwide and 20% of the previously treated cases have the characteristics of multiple drugs-resistance tuberculosis (MDR).

![Estimated TB incidence rates, 2012](image)

**Figure 1.1. Estimated TB incidence rate, 2012.** Color indication represents various incidence rates (estimated new TB cases per 100 000 population per year) (WHO2013).

The overall dynamics of the number of new TB cases per year (indicated by incidence rate) show decline, with the rate of about 2.2% between 2010 and 2011. From 1990 until 2013 there was a 41% reduction in the number of diagnosed cases. It is expected that the number of TB-related deaths registered in 1990 will be halved in 2015. Moreover, the total number of active TB
cases measured by prevalence rate is declining despite the growth of the world’s population. By 2012, prevalence had fallen by 37% globally since 1990 (WHO2013). However, estimations may be imprecise since the majority of the surveys have a sample size of about 50,000 people and cost 1-4 million dollars. It also should be noticed that measuring TB-mortality among HIV-infected patients is problematic and TB infection is often not recorded.

Despite visible improvements in TB treatment, the health threat remains worryingly high. In about 9 out of 10 cases, tuberculosis persists, causing reactivation and resulting in 490,000 deaths per year with 64,000 child deaths. Inefficient medical treatment often results in emergence of multiple drug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB); the latter has been identified in 84,000 of cases, which constitutes about 9% of MDR-TB. The number of MDR-TB cases is especially high in Eastern Europe and central Asia largely caused by temporary shutdown of TB healthcare programs after collapse of the Soviet Union. In a number of these countries, the MDR-TB ratio among new TB-infected patients is 9-32%, and more than 50% of the cases of reinfection belong to the MDR-TB category (Fig. 1.2). Emergence of drug-resistant strains makes total eradication of TB highly unlikely.

**Figure 1.2. Percentage of new cases with MDR-TB.** Color indication represents various mortality rates (WHO 2013).

### 1.1.1 Characteristics of TB

Tuberculosis is transmitted by respiratory fluids of cough and sneeze through the air (Konstantinos 2010). Various species of *Mycobacterium* cause TB, including *M. tuberculosis*, *M. bovis*, and *M. africanum*; they are grouped in the so called *Mycobacterium tuberculosis* complex (Kumar 2007). Most of tuberculosis cases in humans are asymptomatic, resulting in latent forms of
TB, while 1 out of 10 cases progresses to the active form. Usual symptoms of TB include chest pain, chronic cough with blood-tinged sputum, fever, night sweats, weight loss, and fatigue (Dolin 2010). About 90% of the cases show infection of the lungs. Extra-pulmonary TB that occurs in immunosuppressed individuals, 50% of HIV patients and small children, may include other symptoms (Golden and Vikram 2005). Diagnostics of TB include radiology, microscopy, microbiology culture of bodily fluids, and, in the case of latent TB, tuberculosis skin tests (TST) and blood tests.

The main pathogen that causes TB is *Mycobacterium tuberculosis*. *M. tuberculosis* is a small, aerobic nonmotile bacillus that infects primary pulmonary alveolar macrophages (Houben, Nguyen et al. 2006). The granuloma is a complex multicellular structure with infected macrophages in the center, surrounded with T-lymphocytes, B-lymphocytes and fibroblasts. Macrophages play the main role in innate immune response, but in many cases fail to eradicate the infection. Instead, they become reservoirs for *Mycobacterium* replication. Macrophages can develop into multinucleated giant cells and epithelioid cells. T cells represent another member of cell-mediated response while B-lymphocytes initiate adaptive immune response by generation of antibodies. Fibroblasts together with collagen form a fibrous cuff that wall off mycobacteria inside a granuloma. Other cells that participate in granuloma formation include dendritic cells that fulfill the function of antigen representation and NK-cells that trigger lysis or apoptosis of cells with major histocompatibility complex presented on the cell surface (Grosset 2003).

**Figure 1.3. Structure and cellular constituents of the tuberculous granuloma.** Granuloma consists of a tight aggregate of macrophages. Macrophages can fuse into giant multinucleated cells and also
differentiate into foam cells. The center of mature granuloma is necrotic, with multiple apoptotic and necrotic macrophages. Many other cell types are also present, such as dendritic cells, NK cells, neutrophils, T cells and B cells (Ramakrishnan 2012).

The tuberculous granuloma at its most basic is a compact, organized aggregate of epithelioid cells — macrophages that have undergone a specialized transformation to have tightly interdigitated cell membranes that link adjacent cells (Fig.1.3). Epithelioid cells can be highly phagocytic but in some cases do not contain bacteria at all. Granuloma macrophages can also fuse into multinucleated giant cells or differentiate into foam cells, which are characterized by lipid accumulation. Foam cells have been noted to be most frequently located at the rim of the necrotic center of a mature tuberculous granuloma. The consequences of these changes are not well understood, but in general foam cells and multinucleated giant cells have been reported to contain only a few bacteria, if any. Bacteria are most commonly present in the central necrotic areas in which dead and dying macrophages can be seen. Many other cell types also populate the granuloma, such as neutrophils, dendritic cells, B and T cells, natural killer (NK) cells, fibroblasts and cells that secrete extracellular matrix components. Finally, the epithelial cells surrounding the granuloma (not shown) are now thought to participate in its formation also.

It has been assumed that granulomas protect the host from infection; the granuloma may prevent dissemination of the mycobacteria and provide a local environment for interaction of cells of the immune system (Grosset 2003). Recent findings, however show that the granuloma can also be a place for infection of new naïve macrophages that then can escape and seed new granulomas (Ramakrishnan 2012).

Replication of *M. tuberculosis* mostly occurs in macrophages, but can also be detected in dendritic cells, adipocytes, and type II alveolar pneumocytes. Mycobacteria are capable of hijacking a phagosome’s identity and preventing its maturation by blocking its fusion with lysosomes. Details of this ability to manipulate the host are discussed in section 1.4 of this introduction.

**1.1.2 TB treatment**

The modern treatment of drug-susceptible TB includes six months of multi-antibiotic therapy divided in two phases: the first phase consists of two months of four first-line drugs (ethambutol, pyrazinamide, rifampicin, isoniazid) while the second phase involves four months of treatment with isoniazid and rifampicin (Table 1.1). Long duration of therapy and the usage of several antitubercular drugs are crucial in order to completely eradicate TB infection and prevent emergence of drug-resistant strains. The cure rate with this treatment administered under direct observed treatment (DOT) is more than 95% (WHO 2010).

TB treatments face a major challenge. The slow growth and low metabolic rates of *M. tuberculosis* (doubling time of 24 hours) decrease the efficiency of the prescribed drugs. Moreover, *M. tuberculosis* can switch to a dormant non-replicating stage called persistent
(Johnson, Hadad et al. 2009) that significantly increases the time of TB treatment. Other challenges include drug intolerance and toxicity, pharmacokinetic drug-drug interactions, for example with antiretroviral therapy drugs in case of TB-HIV co-infection.

Multiple drug resistant TB (MDR-TB) is detected in 10% of all TB cases and requires prolonged treatment for at least 8 months. Ideally, the total duration of treatment should be at least 20 months for patients with no previous history of MDR-TB infection and 28 months if the patient was already treated for MDR-TB. Therapy should include at least four second-line drugs. In the optimal scenario, the treatment is personalized for each patient with specific parameters depending on the patient’s response to therapy.

Extensively drug resistant TB (XDR-TB) requires the use of third-line anti-TB drugs, which are more expensive and often have more side effects than first-line or second-line drugs. Finally, totally drug-resistant TB (TDR-TB) corresponds to infections with mycobacteria that have acquired resistance to all first and second-line drugs. Treatment of XDR-TB and TDR-TB is extremely challenging and associated with high mortality rates.

Table 1.1. Main anti-tuberculosis drugs in clinical use and their targets (Zumla, Nahid et al. 2013).

<table>
<thead>
<tr>
<th>Drug (year of discovery)</th>
<th>Target</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-line drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoniazid (1952)</td>
<td>Enoyl-[acyl-carrier-protein] reductase</td>
<td>Inhibits mycolic acid synthesis</td>
</tr>
<tr>
<td>Rifampicin (1963)</td>
<td>RNA polymerase, beta subunit</td>
<td>Inhibits transcription</td>
</tr>
<tr>
<td>Pyrazinamide (1954)</td>
<td>S1 component of 30S ribosomal subunit</td>
<td>Inhibits translation and trans-translation, acidifies cytoplasm</td>
</tr>
<tr>
<td>Ethambutol (1961)</td>
<td>Arabinosyl transferases</td>
<td>Inhibits arabinogalactan biosynthesis</td>
</tr>
<tr>
<td>Second-line drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Para-aminosalicylic acid (1948)</td>
<td>Dihydropteroate synthase</td>
<td>Inhibits folate biosynthesis</td>
</tr>
<tr>
<td>Streptomycin (1944)</td>
<td>S12 and 16S rRNA components of 30S ribosomal subunit</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Ofloxacin (1980)</td>
<td>DNA gyrase and DNA topoisomerase</td>
<td>Inhibits DNA supercoiling</td>
</tr>
<tr>
<td>Capreomycin (1963)</td>
<td>Interbridge B2a between 30S and 50S ribosomal subunits</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Kanamycin (1957)</td>
<td>30S ribosomal subunit</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Amikacin (1972)</td>
<td>30S ribosomal subunit</td>
<td>Inhibits protein synthesis</td>
</tr>
<tr>
<td>Cycloserine (1955)</td>
<td>d-alanine racemase and ligase</td>
<td>Inhibits peptidoglycan synthesis</td>
</tr>
</tbody>
</table>
1.2 New anti-tuberculosis drug development

Ideally, a TB drug candidate has to fulfill a set of requirements that includes a fully validated safety profile, a high level of potency to reduce the treatment duration, an ability to inhibit new targets crucial for MDR-TB and XDR-TB treatment, a compatibility with anti-retroviral therapy, and an absence of antagonistic activity with existing or candidate TB drugs. The preferable TB candidate should also target different physiological states of *Mycobacterium*, including the stage of latency (Barry, Boshoff et al. 2009).

Currently, there are a number of candidates in Phase II and Phase III clinical trials and there are many drug candidates at hit-to-lead and lead optimization stages (Fig.1.4). However, there is a significant gap between the preclinical phase of development and Phase I clinical development that requires the attention of researchers in order to compensate for the possible attrition of the more advanced candidates and prevent delays in clinical trials.

![Figure 1.4. Current global pipeline of new antituberculosis drugs.](image)

Repurposing of the drugs that were developed for treatment of other infectious has proved to be beneficial. Examples of repurposed drugs include fluoroquinolones, rifamycins, oxazolidinones and riminophenazines. Compounds targeting the host form another cluster of potential TB drugs that includes drugs that are used for the treatment of rheumatological, cardiovascular, and parasitic diseases. A third group includes drugs with anti-mycobacterial activities that have subsequently been developed as TB drugs candidates, such as bedaquiline, recently approved for therapy in adults with MDR pulmonary TB by the United States Food and Drug Administration (Cohen 2013), the nitroimidazoles PA-824 and OPC67683, SQ109. Several additional novel compounds entered the phase of preclinical development, including the
nitroimidazole TBA-354, the fluoroquinolone DC-159a, the dipiperidine SQ609, the capuramycin SQ641, the benzothiazinone BTZ043 and the caprazene nucleoside CPZEN-45.

1.2.1 Compounds repurposed for TB treatment

Repurposing of the compounds is a common practice in drug discovery. Unlike new chemical entities, repurposed compounds do not require extensive preclinical and clinical trials because many of the drug tests are already completed. It allows shortening the delay required for the drug to enter the market. The major drawback of this approach is the increased risk of emergence of drug resistance as a side effect of previous treatments nonrelated to TB. Repurposed compounds include fluoroquinolones, rifamycins and others (Fig. 1.5).

Fluoroquinolones. The main targets of fluoroquinolones are bacterial DNA gyrases and DNA topoisomerases. Fluoroquinolone compounds are used extensively for the treatment of MDR-TB as a second-line drugs. The idea of moving fluoroquinolones to the first line gained new interest when it was observed that fluoroquinolones reduced the duration of therapy in murine model of TB (Nuernberger, Yoshimatsu et al. 2004). Two fluoroquinolone compounds gatifloxacin and moxifloxacin are currently in Phase III clinical trials that will show whether these compounds could be an effective substitution for ethambutol or isoniazid (Liu, Wang et al. 2010). Notably, fluoroquinolones are frequently used to treat many infectious diseases; consequently, there is a higher risk of emergence of drug resistance in patients that were previously treated for other medical conditions. Finding another drug-like inhibitor of DNA gyrase could be potentially important.

Rifamycins. Rifampicin, which has been a first line TB drug for 40 years, targets the beta subunit of RNA polymerase, preventing transcription. Rifapentine is an analog of rifampicin with the same

Figure 1.5. Drugs repurposed for TB treatment (Zumla, Nahid et al. 2013).
mechanism of action but with a much longer half-life that allow to increase exposure and potentially shorten the duration of treatment (Rosenthal, Zhang et al. 2007). Rifapentine showed results superior to rifampicin in a treatment of latent tuberculosis both in terms of treatment duration shortening and tolerance (Sterling, Villarino et al. 2011). Although rifamycins show high efficiency of TB treatment, there are major drawbacks, particularly induction of cytochromes P450 in the liver, which results in drug-drugs interactions with antiretroviral agents and TB drug candidates such as bedaquiline (Andries, Verhasselt et al. 2005).

**Clofazimine.** Clofazimine is a leprosy drug that can be repurposed to treat MDR-TB (Dey, Brigden et al. 2013). Clofazimine was shown to be effective in the treatment of a mouse model of TB where it was administrated via the aerosol route using microparticles (Verma, Germishuizen et al. 2013). This way of administration can potentially reduce the side effects of clofazimine that include gastrointestinal and dermatological influences. The activity of clofazimine was also observed in murine model of latent TB (Zhang, Sala et al. 2012).

**Oxazolidinones.** Oxazolidinones inhibit protein synthesis by binding to the 23S rRNA in the 50S ribosomal subunit of bacteria. Linezolid is a first-generation oxazolidinone compound. It was shown that linezolid has bacteriostatic activity in vitro and modest activity in mice infected with TB (Fortun, Martin-Davila et al. 2005). Linezolid is effective against MDR-TB and XDR-TB (Fortun, Martin-Davila et al. 2005) (Lee, Lee et al. 2012) although side-effects including neuropathy, myelosuppression, thrombocytopenia, and optic neuritis were observed (Sotgiu, Centis et al. 2012). Four patients were shown to display acquired resistance due two different mutations either in 23S rRNA or rplC genes (Lee, Lee et al. 2012) suggesting that the drug interacts with both ribosomal components.

Another oxazolidinone derivative, sutezolid (also know as PNU-100480), has higher bactericidal activity than linezolid and has entered Phase II clinical trials where it demonstrated significant early bactericidal activity. Sutezolid may have clinical efficacy in humans in a larger Phase 2 trial. (Wallis, Jakubiec et al. 2011).

**Meropenem and clavulanate combination.** *M. tuberculosis* displays resistance to β-lactam antibiotics, including meropenem, as it produces a β-lactamase, BlaC, which efficiently hydrolyses them. It was shown that inhibition of BlaC by clavulanate results in *M. tuberculosis* susceptibility to meropenem (Hugonnet, Tremblay et al. 2009). The mechanism of action of meropenem includes disruption of peptidoglycan biosynthesis (Kumar, Arora et al. 2012). Although both meropenem and clavulanate are approved drugs, successful usage of this drug combination in treatment of TB is hindered by short half-life of meropenem (Kumar, Arora et al. 2012).
1.2.2 New chemical entities for TB treatment

**Bedaquiline.** Bedaquiline, also called TMC207, is a drug approved recently. It inhibits the ATP synthease, leading to decrease of mycobacterial ATP levels (Andries, Verhasselt et al. 2005). Bedaquiline was detected with the use of phenotypic screening, whole-genome sequencing of *M. tuberculosis* mutants, resistant to bedaquiline, displayed the mechanism of action of bedaquiline (Andries, Verhasselt et al. 2005). Resistance was acquired by missense mutations in the atpE gene that encodes the c subunit of ATP synthease. Bedaquiline interferes with the proton translocation step required for ATP generation (Koul, Dendouga et al. 2007). It was shown that bedaquiline is well tolerated by humans since human mitochondrial ATP synthase is 20,000-fold less sensitive to bedaquiline than its mycobacterial counterpart (Haagsma, Abdillahi-Ibrahim et al. 2009). Recent studies also showed interactions with the epsilon subunit of ATP synthase (Biukovic, Gayen et al. 2009).

Advantages of bedaquiline include its activity against dormant *M. tuberculosis* (Koul, Arnoult et al. 2011). Effective killing of dormant bacteria is explained by the finding that de novo ATP synthesis is essential for the viability of non-replicating mycobacteria (Rao, Alonso et al. 2008), which was confirmed subsequently in a murine model of latent tuberculosis (Huitric, Verhasselt et al. 2010). Bedaquiline displays high bactericidal activity both non drug-susceptible and drug-resistant *M. tuberculosis* strains with minimal inhibitory concentrations comparable to isoniazid and rifampicin. Phase II clinical studies show significant increase of patients cured after MDR-TB treatment (Diacon, Pym et al. 2009). A potential advantage of bedaquiline is its long half-life, although accumulation in tissues needs to be taken into account for correct measurements of activity (Lounis, Gevers et al. 2008). Side effects of bedaquiline include arrhythmia induction (Cohen 2013).

**Nitroimidazoles.** Nitroimidazoles, with their known activity against anaerobic organisms, are potential TB drug candidates because anaerobiosis is considered to play important in *M. tuberculosis* survival in latent TB (Migliori, Sotgiu et al. 2013). Metronidazole was the first established nitroimidazole derivative with prodrug qualities, followed by prodrugs PA-824 (Stover, Warrener et al. 2000) and OPC6783 (also known as delamanid) (Matsumoto, Hashizume et al. 2006). PA-824 is activated intracellularly by F420-deazaflavin-depended nitroreductase (Ddn) of *M. tuberculosis* (Manjunatha, Boshoff et al. 2006). The anti-mycobacterial activity of PA-824 is mediated by the generation of reactive oxygen species, including nitric oxide (NO), which are the major effectors in anaerobic conditions acting through poisoning of cytochrome c oxidase (Singh, Manjunatha et al. 2008). The antibiotic effect in aerobic conditions involves inhibition of mycolic acid biosynthesis (Manjunatha, Niranjan-Raj et al. 2009). Similarly, delamanid inhibits mycolic acid biosynthesis and may also induce NO production. Currently, PA-
824 and delamanid are in Phase II and Phase III clinical trials, respectively, despite the side effect such as cardiotoxicity registered for delamanid (Skripconoka, Danilovits et al. 2013).

**SQ109 and other MmpL3 inhibitors.** Researchers have been trying to improve relatively low killing efficiency of ethambutol by synthesizing a more active diamine derivative by means of combinatorial chemistry. The efforts lead to the identification of SQ109 from a library of 60,000 compounds (Lee, Protopopova et al. 2003). It was shown that SQ109 targets MmpL3, a membrane protein of the resistance, nodulation and division (RND) family (Tahlan, Wilson et al. 2012). MmpL3 is known to transport trehalose monomycolate into the cell envelope; inhibition of MmpL3 by SQ109 thereby results in inhibition of mycolic acid biogenesis.

**Benzothiazinones.** Benzothiazinones display nanomolar bactericidal activities on mycobacteria growing in vitro and in ex vivo models (Makarov, Manina et al. 2009). In murine TB models benzothiazinone BTZ043 showed efficacy comparable with that observed with isoniazid and rifampicin. It is also active against MDR-TB and XDR-TB (Pasca, Degiacomi et al. 2010) with no significant antagonism with other anti-tubercular compounds including rifampicin, ethambutol, bedaquiline, isoniazid, PA-824, moxifloxacin, and SQ-109 (Lechartier, Hartkoorn et al. 2012). BTZ043 targets decaprenylphosphoryl-beta-d-ribose 2’-epimerase (DprE1) (Batt, Jabeen et al. 2012). The role of DprE1 is to catalyze the conversion of decaprenyl-phosphoryl-d-ribose to decaprenyl-phosphoryl-d-arabinose, which is a precursor of mycobacterial cell wall components arabinogalactan and lipoarabinomannan.

**1.3 Target identification in drug discovery**

Target identification includes identification of the target pathways and actual molecular interactors such as proteins, even though the exact difference between these two levels of targets is often difficult to distinguish. There are two main approaches to identify the target pathways and interactors of drug-like compounds: genetic interaction methods and direct biochemical methods that are backed up by computational inference methodology. Biochemical methods are considered the most straightforward. They include identification of the interacting protein by attaching a cross-linker to the molecule, perform a “fishing” procedure, subsequent washing and purification of the covalent complex. Next stage includes labeling of identified protein or small molecule of interest, followed by a step of incubation of the protein with the small molecule and direct measurements of binding (Burdine and Kodadek 2004). Computational inference methodology may be used for generation of target hypothesis by pattern recognition analysis of the small molecule and referenced protein targets (Weinstein, Myers et al. 1997, Young, Bender et al. 2008). The hypothesis is afterwards confirmed experimentally by measuring molecular interactions in biochemical assays to establish mechanistic hypotheses. The target pathways are confirmed experimentally with the use of genetic methods (Fomina-Yadlin, Kubicek et al. 2010).
Hypothesis for the mechanism of action can be generated by analysis of changes in gene expression patterns in the presence or absence of compound. This approach allows the identification of potential target pathways, which in turn can be narrowed down to molecular targets with the use of a combination of \textit{in silico}, biochemical and genetic methodology. Determining bacterial targets involves selection of mutants, either arising spontaneously or induced by mutagenesis, that are resistant to the compound and identification of the affected gene via sequencing.

Naturally, target identification approaches are not mutually exclusive; the majority of drug discovery campaigns use a combination of biochemical and genetic methods to maximize precision of target hypothesis. Generally, integration of multiple complementary approaches is required to fully solve the problem of target identification.

1.3.1 Genetic interaction and genomic methods for drug target discovery

Drug discovery based on genetic or genomic methods relies on manipulations with DNA and RNA that affects the whole \textit{in vivo} system. Hypothetical targets are generated by exploring phenotypes of genetically altered organisms. Researchers use gene knockouts, RNAi (Boutros and Ahringer 2008) and small molecules with the known activity to change the functional activity of supposed targets. For example, a knockdown that phenocopies a compound’s effect suggests involvement of the depleted gene product in the compound’s mechanism of action and demonstrates potential chemical-genetic interactions (Fig. 1.6a). The absence of a clear target hypothesis may be compensated by generation of multiple weak target hypothesis and their subsequent approval/disproval.

Yeast is an example of well-known model with established genetics methods for target identification. In yeast, interactions of small-molecule with specific genetic loci can be revealed by generation of recombinant strains by mating. The method involves subsequent analysis of the recombinant strains that are resistant or sensitive to specific small molecule (Perlstein, Ruderfer et al. 2007) (Fig.1.6b). Patterns of polymorphisms indicate potential genetic targets. Another approach includes transformation of the clones with molecularly barcoded libraries of open reading frames. Clones fitness is analyzed with the use of microarrays to detect small molecule-resistance or increased sensitivity (Pierce, Fung et al. 2006).

The use of the yeast and other model organisms may not be effective, since direct translation to human biology may not always be achieved due to lack of conservation of some genetics pathways involved in pathology. Development of modern genetic engineering methods such as CRISPR-derived technology may be beneficial to studies of drugs mechanism of action in mammalian systems.
Figure 1.6. Illustrations of yeast genomic methods for target-identification and mechanism-of-action studies. (a) A panel of viable single-gene deletions is tested for small-molecule sensitivity; mechanisms are interpreted by comparing interactions to double-knockout strains. (b) Different strains of diploid yeast are mated to form F1 recombinants, and meiotic progeny are subjected to small molecules; segregation frequencies allow mapping of small-molecule sensitivity to genetic loci. (c) A recessive small molecule–resistant mutant is transformed with a wild-type open reading frame library; transformants obtaining a wild-type copy of the mutant gene are selectively sensitive to small molecules, quantified by microarray (Schenone, Dancik et al. 2013).

Another genetics-based technique for target identification includes comparison of results from small-molecule and perturbations by RNAi. The technique is similar to generation of knockouts but in this case gene expression is altered by the use of RNAi. Identified similarities of phenotypes between small-molecules and RNAis indicate the possible mechanisms of action. RNAi screenings can be established on a genome-wide scale in order to find analogous phenotypes between small molecules and RNAi. (Fig. 1.7a) Once primary data are obtained, a set of RNAi reagents can be narrowed down for the precise indication of pathway members involved in phenotypic changes. (Fig. 1.7b) (Guertin, Guntur et al. 2006). Identification of the exact molecular targets can be then facilitated by the use of biochemical and in silico methods.

The advantages of RNAi methods include the ability to detect phenotypic changes in a more physiologically relevant environment using mammalian cells. However, genetic perturbations cannot always phenocopy the effect of a small molecule (Knight and Shokat 2007) due to the risk of genetic compensation or multiple effects generated by the small molecule. Additionally, the drug might not always photocopy a functional knockdown; instead, it might titrate molecular interactions and induce a dominant negative effect.
Figure 1.7. Illustration of RNAi based for methods of target identification and mechanisms of action studies. a) In one implementation, phenotypes from genome-wide RNAi are compared to those induced by a small molecule of interest; phenocopy of the small-molecule effect by RNAi provides evidence that the gene product is a small-molecule target. (b) When prior evidence suggests a particular target pathway, focused sets of RNA reagents can help to generate mechanistic hypotheses (Schenone, Dancik et al. 2013).

Finally, analysis of gene expression profiles to determine a compound’s mechanism of action blurs the line between genetic techniques and computational approaches. For example, a recent study used transcription profiling data from the Connectivity Map (Lamb, Crawford et al. 2006) and described a weighting scheme to classify and order lists of genes across various cell lines in an attempt to develop a prototype ranking list (Iorio, Bosotti et al. 2010). Such an approach has its limits, since it relies on accurately annotated activities of small-molecules, but the continuously growing datasets of gene profiling will facilitate the merge of genetic and bioinformatical methods.

1.4 Mycobacterium marinum as a pathogen model for mycobacterial infection

1.4.1 Selection of a Mycobacterium model for drug screening purposes

Mycobacterium tuberculosis, as a pathogen that causes tuberculosis in humans, seems to be the most straightforward choice for studying mechanisms of TB infection and screenings of
antitubercular compounds. However, such a direct approach has its drawbacks. One of the challenges is the problem of biosafety. *M. tuberculosis* is a category 3 pathogen and possesses significant danger for the research staff (Cosma, Sherman et al. 2003). Strict safety requirements reduce the numbers of research facilities that can perform TB-related experiments. Secondly, growth and metabolic rates of *M. tuberculosis* are very low. Its doubling every 22 hours in liquid culture makes experiments time-consuming, which is especially unwelcome in high-throughput screenings. Additionally, many drugs screens showed low hit to lead ratios that raises the demand of using alternative approaches and alternative pathogen systems in particular (MacGurn and Cox 2007).

To avoid these constraints, a set of alternative *Mycobacterium* models have been established. The most recognized of them include *Mycobacterium bovis* (BCG strain), *Mycobacterium smegmatis* and *Mycobacterium marinum*. Among them, *M. bovis* is the only *Mycobacterium* that belongs to the TB complex. BCG is the conventional strain of *M. bovis* attenuated by serial passage in the laboratory. BCG has similar to *M. tuberculosis* growth rates, on the other hand, unlike *M. tuberculosis*, BCG is a Category 2 pathogen. However, BCG growth rate is similarly low, as of *M. tuberculosis*.

*M. smegmatis*, a distant relative of *M. tuberculosis* that dwells in soil solves the problem of slow growth with a doubling time of four hours and colony generation in two to three days. Moreover, it is harmless to humans, and requires only Category 1 biosafety level. It is also convenient in terms of genetic manipulations. Although, the model has its drawbacks, such as already mentioned relative evolutionary distance of *M. smegmatis* and *M. tuberculosis* and lack of TB-like pathogenesis which make it problematic to search for infection of specific activities of compounds.

*M. marinum* has an intermediate role between BCG strains and *M. smegmatis* models. On one hand, it provides relative ease of cultivation and manipulation, and relatively low Category 2 biosafety level, on the other hand, it is capable to induce TB-like pathogenesis important for antitubercular drug-screenings. *M. marinum* is also the species of *Mycobacterium*, that is the closest genetically to Mtb complex. Taken together, it makes *M. marinum* an efficient bacterial model of *M. tuberculosis*.

Studies show that *M. marinum* is capable to induce TB-like pathogenesis (Table 1.2). Indeed, *M. marinum* infection leads to granulomas induction in humans resulting in pathogenesis indistinguishable from *M. tuberculosis* dermal granulomas that fit morphological criteria of tuberculosis such as the presence of epithelioid cells surrounded by a lymphocytic cuff, frequently with a central necrotic core (Travis, Travis et al. 1985) (Dinning and Marston 1985). In ectothermal hosts, *M. marinum* produces complete systemic tuberculosis-like disease. Natural host organisms for *M. marinum* include fish and frogs that also can be a subject of TB research (Cosma, Swaim et al. 2006). Particularly, leopard frogs were shown to develop lifelong asymptomatic
infection, the property similar to the majority of infected humans and are found to bear highly organized non-caseating granulomas (Ramakrishnan, Valdivia et al. 1997), (Bouley, Ghori et al. 2001) [8]. Fish ectotherms, like medaka, goldfish and zebrafish are prone to producing caseating granulomas that show resemblance to active caseating human tuberculosis (Talaat, Reimschuessel et al. 1998, van der Sar, Abdallah et al. 2004, Swaim, Connolly et al. 2006).

Despite being primarily a pathogen of ectothermal animals, M. marinum also can be used as a pathogen model of mammalian hosts. For example, the infection mouse model has been used for over 40 years and is still in occasional use (Clark and Shepard 1963, Robinson, Wolke et al. 2007). However, in mice infected with M. marinum, bacterial numbers decrease with time, contrary to what is observed in majority of natural hosts. The possible explanation probably involves the differences in temperature of the host models. Systemic infection could be induced by an intravenous infection that result in limited non-caseating epithelioid granulomas in liver and spleen (Robinson, Wolke et al. 2007). The “mouse tail” infection model also allows surpassing temperature restrictions due to lower temperature of the mouse tail. In this model M. marinum proliferates and triggers Esx-1-dependent formation of caseating granulomas similar to those formed in human tuberculosis, and bone deterioration reminiscent of skeletal tuberculosis (Carlsson, Kim et al. 2010).

Overall similarity of M. marinum and M. tuberculosis antigen determinants is confirmed by the studies that showed that immunization with M. marinum protected from subsequent M. tuberculosis challenge (Collins, Montalbine et al. 1975). Granuloma formation includes some minor differences. For instance, in contrast to M. tuberculosis, M. marinum induces granulomas with a low lymphocytes content (Bouley, Ghori et al. 2001, Swaim, Connolly et al. 2006), although it was shown that fish lymphocytes are important for the restriction of mycobacterial growth; adult rag1-mutant zebrafish, which lack T and B lymphocytes are hyper-susceptible to M. marinum infection, similar to M. tuberculosis-infected rag1 mutants (Saunders, Briscoe et al. 2004).

Table 1.2. A comparison of three mycobacterial models to M. tuberculosis. The similarities and differences among M. smegmatis, BCG and M. tuberculosis are highlighted (Shiloh and Champion 2010)
1.4.2 Mycobacterial infection – entrance, persistence and escape.

Mechanisms of infection of *M. tuberculosis* and *M. marinum* display a high level of phenotypic similarity (Fig.1.8). Indeed, it was shown that both inside mammalian macrophages and fish monocytes, live but not heat-killed *M. marinum* localizes to a non-acidified phagosomal compartment that excludes the vacuolar proton ATPase, a trait known to be one of the key intracellular features of *M. tuberculosis* infection (Barker, George et al. 1997, El-Etr, Yan et al. 2001). Presumably, prevention of acidification protects pathogenic bacteria from phagolysosome-associated killing and possibly modulates the host adaptive immune response by changing antigen presentation (Russell, Mwandumba et al. 2002).

![Figure 1.8. Schematic representation of the fate of pathogenic *M. marinum* during establishment, maintenance and cell-to-cell spreading of an infection in *D. discoideum*. Uptake of *M. marinum* by *D. discoideum* results in the formation of a phagosome that obtains marker vacuolar H+-ATPase. Further maturation is bypassed (indicated by a bold arrow), the level of vacuolar H+-ATPase becomes undetectable and delivery of cathepsin D (interrupted arrows) is blocked. The vacuole gradually accumulates vacuolin (*D. discoideum* protein that belongs to flotillin family) on its cytoplasmic surface (12–37 hours post infection, hpi). Mycobacteria proliferates (12–37 hpi) the vacuolin coated and p80 (predicted copper transporter) positive-compartment ruptures and the bacteria are released into the host cytosol (37–43 hpi). The presence of the pathogen protein MAG24-1 and the host protein vacuolin B are crucial for efficient establishment and/or maintenance of the replication niche. A small percentage of mycobacteria follows traditional phagosomal pathway (Hagedorn and Soldati 2007).](image-url)
Although the majority of *M. tuberculosis* mutants that fail to facilitate phagosomal maturation arrest have significant restrictions in intracellular growth, there is an interesting class of mutants that is able to multiply as well or better in cultured macrophages despite phagosomal maturation, suggesting that phagolysosome fusion arrest is not an absolute requirement for *M. tuberculosis* survival, at least in naïve cultured macrophages (Pethe, Swenson et al. 2004) (Stewart, Patel et al. 2005).

The intracellular localization of *M. marinum* and *M. tuberculosis* may vary. For instance, it was observed in macrophage-like cell lines that a fraction of *M. marinum* can escape from the phagosome into the cytosol and develop actin-based motility (Stamm, Morisaki et al. 2003). Some electron microscopy studies suggested a possibility of phagosomal escape by *M. tuberculosis* (McDonough, Kress et al. 1993). *M. tuberculosis* was shown to be capable of escaping from the phagosome in cultured dendritic cells in studies assessed by cryo-electron microscopy (van der Wel, Hava et al. 2007). Overall *M. tuberculosis* escape data remain controversial and the view of *M. tuberculosis* staying in the phagosome was the most widely approved (Jordao, Bleck et al. 2008). However more recent data indicates that *M. tuberculosis* escape does occur. It is mediated by ESX-1 and results in necrotic cell death of the host (Simeone, Bobard et al. 2012). Apparently, induced cytotoxicity was the reason why cytosolic *M. tuberculosis* was not observed frequently by microscopic analysis.

The behavior of *M. marinum* in the cytosol has its own peculiar features. While in *M. tuberculosis* infection actin based motility has not been observed, about 20% of *M. marinum* bacteria obtain motility by the WASP family-dependent activation of actin polymerization, sharing similarities with the mechanism of motility employed by *Shigella* (Stamm, Pak et al. 2005). Phagosomal escape, subsequent cytosolic actin polymerization and cell–cell spreading can be abrogated by mutations in the *M. marinum* ESX-1 secretion system (Gao, Pak et al. 2006). Notably, research groups that do observe *M. tuberculosis* phagosomal escape also mark dependence on ESX-1 (van der Wel, Hava et al. 2007). Stamm and Brown suggested that actin polymerization is used by *M. marinum* at a different stage of infection, prior to granuloma formation, to cross the epithelial barrier after host ingestion or for transmission from unicellular organisms to vertebrate hosts – both types of transmission that are not used by *M. tuberculosis* (Stamm and Brown 2004). Apparently, actin-based motility is used by *M. marinum* in a limited set of conditions and is a representation of the unique and expanded functional repertoire in comparison to *M. tuberculosis*.

Actin is also actively involved in bacterial escape from the host. It was shown, that *M. tuberculosis* and *M. marinum* can leave the host Dictyostelium discoideum via non-lytic ejection through an actin-based structure called the ejectosome (Hagedorn, Rohde et al. 2009).
1.4.3 *M. marinum* virulence determinants in comparison to *M. tuberculosis*

*M. marinum* and *M. tuberculosis* share similar mechanisms of virulence on a genetic level. First transposon-based mutagenesis screens showed that 70-85% *M. marinum* virulence genes have orthologues in *M. tuberculosis* (Ruley, Ansed et al. 2004). Divergence may occur due either to the cell assay specificity, to the differences in the biology of infection, or to the presence of a functional non-orthologue equivalent. More detailed mutant analyses show that *M. marinum* and *M. tuberculosis* share a majority of orthologous virulence determinants.

Naturally, there are some species-specific differences in virulence gene repertoire. For example, cell surface sulfated lipids encoded in a multi-gene locus in *M. tuberculosis*, such as Sulfolipid-1, are not present in *M. marinum* but supposedly contribute to virulence (Bhatt, Gurcha et al. 2007). In mice it was shown that accumulation of the sulfolipid intermediate SL1278 in mmpL8 *M. tuberculosis* results in attenuation of virulence, although the blockage of the upstream synthesis of sulfolipids show no distinguishable phenotype (Rousseau, Turner et al. 2003). The effect of SL1278 is most likely due to modulation of the host immune system (Domenech, Reed et al. 2004).

Rv0987-9 is another virulence locus supposedly specific to *M. tuberculosis*. It was shown to affect macrophages but not in *in vivo* infection (Domenech, Reed et al. 2004).

The VirS locus is the only divergent *M. tuberculosis*-specific gene with a distinct *in vivo* phenotype. It was shown that virS locus mutation reduces bacterial loads in the spleen by 800-fold in a guinea pig infection model but did not affect lung infection (Singh, Gupta et al. 2005).

Other studies also display high level of virulence similarity between these two *Mycobacterium* species. The Rubin research team identified 126 genes in *M. tuberculosis* that were necessary for survival of the bacteria in macrophages (Rengarajan, Bloom et al. 2005) and 194 important for *in vivo* proliferation (Sassetti and Rubin 2003). Among them only 3% were specific for *M. tuberculosis*. Overall findings suggest that *M. tuberculosis* and *M. marinum* share a common program of pathogenesis with a few refinements emerging due to horizontal gene transfer over the course of evolution.
Table 1.3. Comparison of selected virulence determinants of *M. marinum* and *M. tuberculosis* (Tobin and Ramakrishnan 2008).

<table>
<thead>
<tr>
<th></th>
<th><em>M. marinum</em></th>
<th>Mutant phenotype</th>
<th><em>M. tuberculosis</em></th>
<th>Mutant phenotype</th>
<th>Cross-species complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall lipids</td>
<td></td>
<td>+</td>
<td>+</td>
<td>Attenuated, Rifampicin hypersusceptibility</td>
<td>+</td>
</tr>
<tr>
<td><em>kaeB</em> (mycolic acid structure)</td>
<td></td>
<td>Attenuated, Rifampicin, defensin hypersusceptibility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDIM</td>
<td>Altered stereochemistry, biosynthetic pathway, transporters present</td>
<td>ND</td>
<td>+</td>
<td>Attenuated</td>
<td>ND</td>
</tr>
<tr>
<td>TDM</td>
<td>Biosynthetic pathway present</td>
<td>ND</td>
<td>+</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>PGL</td>
<td>Biosynthetic pathway present</td>
<td>ND</td>
<td>Some strains (e.g. W-Beijing)</td>
<td>Presence of PGL correlates with hypervirulence</td>
<td>ND</td>
</tr>
<tr>
<td>SL1</td>
<td>Carotenoids</td>
<td>−</td>
<td>NA</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td><em>CtnB</em></td>
<td>+</td>
<td>Attenuated</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Cmp</em></td>
<td>+</td>
<td>Attenuated, Rifampicin hypersusceptibility</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Erp</em></td>
<td>+</td>
<td>Attenuated</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>ESX secretion</td>
<td>ESX-1</td>
<td>+</td>
<td>Attenuated, phagolysosome fusion, granuloma formation, haemolysis</td>
<td>Attenuated, altered cytokine profile, phagolysosome fusion and others</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ESAT-6</td>
<td>+</td>
<td>Attenuated</td>
<td>Attenuated</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CFP-10</td>
<td>+</td>
<td>Attenuated</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>EspA</td>
<td>Probable, but orthologue unclear</td>
<td>ND</td>
<td>Attenuated</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>EspB</td>
<td>+</td>
<td>Attenuated</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ESX-S</td>
<td>+</td>
<td>Attenuated</td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

1.4.4 Mycobacterial cell wall structure and cell wall mutants

The structure of the mycobacterial cell wall is one of the reasons why it is so difficult to design anti-tubercular drugs. It is a distinguishing feature of all *Mycobacterium* species. The mycobacterial cell wall is much thicker than many other bacterial species and contains a highly hydrophobic mycolate layer and a peptidoglycan layer held together by the polysaccharide arabinogalactan (Fig. 1.9). The unique characteristics of the highly impermeable mycobacterial cell wall explain the overall hardness of the *Mycobacterium* genus and its low susceptibility to antibiotics. Therefore, the biosynthetic pathways of cell wall components are crucial potential targets for new drugs for tuberculosis.

A remarkable manifestation of cell wall structure is the cording phenotype. The cording phenomenon in virulent mycobacteria was observed more than a century ago (Koch, 1882). It was noted that non-pathogenic mycobacteria lack cording morphology. The same effect was observed for a number of *M. tuberculosis* mutants with growth defects due to changes in cell wall structure. Mutant generation in *M. marinum* revealed similar correlation between cording and virulence.
Figure 1.9. The schematic structure of mycobacterial cell wall. Depicted here is one of the current views of the mycobacterial cell wall. The cell wall is mainly composed of a large cell-wall core or complex that contains three different covalently linked structures (peptidoglycan (grey), arabinogalactan (blue) and mycolic acids (green)). The covalent linkage of mycolic acids results in a hydrophobic layer of extremely low fluidity. This layer is also referred to as the mycomembrane. The outer part of the mycomembrane contains various free lipids, such as phenolic glycolipids, phthiocerol dimycocerosates, cord factor or dimycovyltrehalose, sulpholipids and phosphatidylinositol mannosides, that are intercalated with the mycolic acids. Most of these lipids are specific for mycobacteria. The outer layer, which is generally called the capsule, mainly contains polysaccharides (glucan and arabinomannan) (Abdallah, Gey van Pittius et al. 2007).

Strains with mutations in cell wall components provided significant help in understanding mycobacterial cell wall structure and its influence on cell functioning. *M. marinum kasB* mutant is one of these identified mutants. KasB mutants synthesize mycolic acids that are two-to-four carbons shorter than those synthesized by wild-type *M. marinum*, show reduced levels of keto-mycolates and have cording defects (Gao, Laval et al. 2003). KasB mutants also display growth attenuation and probably have alteration in cell wall permeability that renders them more sensitive to a number of lipophilic compounds and host defense molecules (Gao, Laval et al. 2003). The analogous *M. tuberculosis* deletion results also display significant changes in cell wall composition and a high level of attenuation in a mouse model (Rao, Fujiwara et al. 2005).
The *erp* locus, characterized by targeted deletion, is responsible for expression of a cell surface protein (Berthet, Lagranderie et al. 1998). Mutation of the *erp* gene in *M. tuberculosis* and *M. marinum* results in reduction of bacterial fitness in both cultured macrophages and animal models of infection and results in hypersusceptibility to lipophilic antibiotics (Cosma, Klein et al. 2006). Zebrafish embryo experiments show that the *erp* mutant is successfully phagocytosed by macrophages, but is unable to survive inside the host. (Cosma, Klein et al. 2006). Depletion of macrophages rescues the attenuated phenotype of *erp* mycobacteria, showing that this virulence factor is specific for macrophage defenses (Clay, Davis et al. 2007).

The *M. marinum* locus *iipA* is another virulence factor, the corresponding mutation affects susceptibility to antibiotics and cell permeability through changes in cell wall structure (Gao, Pak et al. 2006). *M. marinum* *iipA* and *iipB* are orthologues of *M. tuberculosis* Rv1477 and Rv1478, both orthologues contain conserved NLPC_p60 domains that are shown to mediate peptidoglycanase activity. Mycobacteria with an *iipA* mutation display cording defects and attenuated pathology and intracellular survival. Attenuation was confirmed by infection of zebrafish with high numbers of *iipA* mutant mycobacteria. The *M. marinum* *iipA* phenotype can be rescued by complementation with the *M. tuberculosis* orthologue; this suggests that the genes have conserved functions.

In *M. tuberculosis*, specific cell wall lipids such as phthiocerol dimycocerosate (PDIM), trehalose dimycolate (TDM) and a polyketide synthase-derived phenolic glycolipid (PGL) are important determinants of virulence (Reed, Domenech et al. 2004) (Glickman, Cox et al. 2000, Gao, Pak et al. 2006). Cell wall lipids were shown to be involved in cording and display interaction with the host defense response. Although all discovered *M. tuberculosis* cell wall virulence lipids are predicted equivalents in *M. marinum*, extensive functional and biochemical analyses of these genes were not performed.

### 1.4.5 ESX secretion systems and their substrates

The ESX secretion systems play important roles in pathogenesis across a wide variety of Gram-positive bacteria (Abdallah, Verboom et al. 2006) and are present in mycobacteria as well. Particularly, ESX-1 involvement is crucial in mycobacterial infection (Fig.1.10). *M. tuberculosis*, *M. marinum* and *M. bovis* mutants that lack the ESX-1 system of the downstream substrates show attenuation in both cultured macrophages and in animal infection models. The majority of the ESX-1 secretion system is located in the RD1 locus. RD1 deletion results in defects in cell-to-cell transmission, changes in cytokine profiles, and an inability to inhibit phagolysosome fusion (reviewed in (Abdallah, Gey van Pittius et al. 2007)).

ESX-1 may be important in phagosome escape for *M. marinum* and BCG (Gao, Guo et al. 2004). ESAT and CFP-10 are present in the ESX-1 locus and show co-dependency for secretion, similar co-dependency in secretion is observed for the EspA gene that lies outside of the
ESX-1 locus (Fortune, Jaeger et al. 2005). In *M. marinum* it was observed that the lack of EspB substrate of the ESX-1 system participates in attenuation and has a possible contribution to defects in phagosome maturation arrest (McLaughlin, Chon et al. 2007).

**Figure 1.10. Model of known interactions (a) and predicted localizations (b) of the ESAT-6–CFP-10 heterodimeric complex.** The secretion of Rv3616c (also known as EspA) is co-dependent on the presence of the ESAT-6–CFP-10 complex. However, there is no formal evidence that these proteins form a larger complex. The ESAT-6–CFP-10 complex is recognized by the FtsK/SpoIIIE-like protein Rv3871, which binds the carboxy (C)-terminal tail of CFP-10. Rv3871 itself is associated with the inner membrane (IM) by its interaction with Rv3870. The translocation channel in the IM is probably formed by Rv3877, which has many transmembrane domains, although it is unknown which protein (or proteins) forms the channel in the mycomembrane (MM). The AAA+ chaperone-like protein Rv3868 could be involved in the biogenesis of the secretion machinery. The function of the subtilisin-like protease MycP1 is essential, but it is not known why, as no protein has been identified that is cleaved upon secretion by ESX-1. Gene families that are also present in other ESX gene clusters are shown in colours, whereas ESX-1-specific genes are shown in dark grey. A question mark indicates that the mycomembrane channel has not yet been identified. (Abdallah, 2007).

Notably, in both *M. tuberculosis* and *M. marinum* infection, components of ESX-1 secretion are essential for phagosome maturation arrest, yet the *M. bovis* BCG strain with deletion of the ESX-1 locus is still able to arrest phagolysosome fusion (MacGurn and Cox 2007). Unlike in *M. marinum*, in *M. tuberculosis* EspB is secreted in the absence of CFP-10 (McLaughlin, Chon et al. 2007). In *M. marinum*, EspB, ESAT-6 and CFP-10 are all co-dependent for secretion (Xu, Laine et al. 2007).

Other ESX secretion systems do not display a high level of redundancy and their substrates may also play roles in mycobacterial pathogenesis. The *M. marinum* esx-5 locus has an
orthologue in *M. tuberculosis*. Esx-5 is involved in secretion of PPE41, a member of a family of mycobacterial genes that include the PE and PPE gene families. The exact function of these families, which comprise 10% of mycobacteria virulence genes, is not well understood (Abdallah, Verboom et al. 2006).

1.5 Single-cell models to study host-pathogen interactions

Research on human infections struggles with the incredible amount of factors scientists have to deal with. Indeed, the human body is a complex conglomerate of about 37.2 trillion cells with characteristics varying significantly in space and time. Cells are exchanging enormous amount of information at molecular, cellular, and tissue levels of structural organization. The sheer abundance of signals makes the dissection of each separate aspect of human biology a challenging issue. The complexity of the system reaches another level when the organism has to confront external pathogenic stimuli such as invasion of pathogenic microorganisms.

In order to cope with the problem of excessive complexity, steps of simplification and standardization have to be taken. It gave rise to research based on alternative model organisms and *in vitro* studies. One way to reduce the noise generated by multiple factors is to switch from a whole organism to a single cell. Single-cell and cultured-cell models lie in between *in vitro* and multicellular *in vivo* studies and provide a useful compromise between model complexity and precision. One has also to admit that the usage of unicellular models has its drawbacks such as the potential to overlook multicellular interactions and the possible risk of fundamental differences in responses due to different genetics in the case of protozoan models. In any case, host-pathogen research always involves a trade between modeling precision, complexity, ease of cultivation and other factors. Diversity of the approaches allows the most complete and profound understanding of infection.

Depending on the type of infection, host choice may vary. Monitoring tuberculosis at an organismal level in mammalians requires the use of mice, guinea pigs, rabbits or non-human primates. This approach allows the detection of complex immune responses, such as granuloma formation. Notably, specific characteristics of granulomas may significantly vary across species. For example, in the mouse model non-caseating granulomas are being produced and the necrosis level is minimal (McMurray 2001). The highest similarity to human tuberculosis is observed in primates, as expected. However, this model has its drawbacks including high cost, extensive use of laboratory space, and ethical problems. Alternatives to mammalian hosts for TB research involve the use of *Drosophila melanogaster* (Hoffmann 2003), *Caenorhabditis elegans* (Couillault and Ewbank 2002) that are focused on studying conserved innate immune mechanisms and *Danio rerio*, which are suited both for innate and adaptive immunity studies (Prouty, Correa et al. 2003).
In case of TB research focused on the single-cell level, it is necessary to select a host with macrophage-like properties since the macrophages are the primary target of *M. tuberculosis*. The main characteristics of macrophages include enhanced ability to phagocytose and kill bacteria. Unicellular host models that fulfill these criteria may be divided into two categories: (1) professional phagocytes of an immune system plus their cultivated derivatives that eradicate infection and (2) free-living protozoa that feed on bacteria via the use of phagocytosis. The latter approach is less frequently used but at the same time could provide some fresh insights and ideas, which are increasingly needed in the current field of tuberculosis research.

Free-living amoebae are one example from the second category. Like a macrophage, an amoeba possesses characteristics of a professional phagocyte. This results in a high degree of functional conservation in defense mechanisms against microbes between amoebae and macrophages (Steinert and Heuner 2005). Similarity may be explained by the fact that the common ancestors of amoeba and macrophages served as a natural reservoir and a training field for pathogens, and that it developed defense mechanism against them that were later utilized by the immune system within the context of infection. To give a more precise answer to the question of host model selection, one could name two species of amoebae: *Dictyostelium discoideum* and *Acanthamoeba castellanii*, models that are already well established in other areas of biological research, particularly in the fields of cell motility and phagocytosis. Naturally, the use of amoebae for TB research does not exclude use of macrophages and their cultivated derivatives as a validation step.

Ease of cultivation of amoebae and genetic tractability (in the case of *D. discoideum*) makes them a potentially attractive system for drug screenings, cytotoxicity assays, and mechanism of action studies. *D. discoideum* could be a useful addition to pharmaceutical screens, although not a complete substitution. Ignoring the mammalian immunity counterpart can run the risk of discovering drugs that are amoeba-specific. Tandem usage of both of systems would be the most productive.

1.5.1 *Dictyostelium discoideum* as a host model for infection

*D. discoideum* (Fig.1.11) provides ease of cultivation and a convenient host model for a variety of pathogenic bacteria and fungi (Steinert and Heuner 2005). Ease of genetic manipulation is a well-recognized feature of *D. discoideum*, with the arsenal including homologous gene replacement, random insertion mutagenesis, multiple-gene knockouts and RNA interference techniques (Steinert and Heuner 2005). The detection of mutant phenotypes can be fast and sensitive due to its haploid genome. The *D. discoideum* genome is fully sequenced and contains a large variety of proteins with mammalian orthologues, displaying a high degree of functional conservation. Notably, about 33 *D. discoideum* proteins are homologous to human proteins involved in diseases (Eichinger, Pachebat et al. 2005). In addition, there are 24 classes of protein kinases contained in the *D.discoideum* kinome. Researchers established a large set of stable
knockout cell lines and cell lines with overexpressed genes that are very helpful for the investigation of signaling pathways and protein function (http://www.dictybase.org).

![Phylogenetic tree of *D. discoideum*](image)

**Figure 1.11. Phylogenetic tree of *D. discoideum***. *D. discoideum* is a member of the Amoebozoa, a taxon that is basal to the Fungi-Metazoa branch (Eichinger, Pachebat et al. 2005).

The *D. discoideum* model facilitates investigation of strategies of bacterial pathogens to escape phagocytic killing. The outcomes of infection were analyzed by a number of *D. discoideum* infection assays, transcriptomic and proteomic studies (Farbrother, Wagner et al. 2006, King and Insall 2009, Shevchuk, Batzilla et al. 2009, Urwyler, Nyfeler et al. 2009).

An example of an assay optimized for studying host-pathogen interactions is the *D. discoideum* plaque assay. Such an assay reveals potential virulent characteristics of bacteria like the ability to evade amoeboid killing or displaying toxic properties for the host. However, the assay does not discriminate between multiple parameters, such as extracellular toxicity, inhibition of phagocytosis and intracellular killing. The use of plaque assays allowed identification of *P. aeruginosa*, *B. cenocepacia*, *K. pneumoniae* and *V. cholerae* with possibly altered resistance to intracellular killing, although additional assays need to be used for discrimination of parameters mentioned above. (Pukatzki, Kessin et al. 2002, Benghezal, Fauvarque et al. 2006, Pukatzki, Ma et al. 2006, Aubert, Flannagan et al. 2008).

Another type of assay, well established in *D. discoideum* is the phagocytosis assay. It measures the increase of resistance or susceptibility of host cell mutants to phagocytosis-related infection. Intracellular growth rates are compared to the well-defined control strains. Phagocytosis assays can also analyze mutant bacterial strains by comparison to the well-characterized host cell strain. Phagocytosis assays showed that the uptake of different bacterial species varies significantly. For example, uptake of pathogenic *L. pneumophila* is very low compared to non-
pathogenic *E. coli* (Skriwan, Fajardo et al. 2002). Notably, entry of Legionella is by macropinocytosis (Watarai, Derre et al. 2001). The level of uptake is characterized by the CFU counts of the first time point of the bacterial growth curve. Therefore the assay can measure the overall intracellular growth/killing of bacteria if the levels of uptake in analyzed samples are similar.

Furthermore, a large arsenal of genetic manipulation techniques is available for *D. discoideum* including biological markers, GFP-tagged proteins, and other visualization techniques. This methodology enables a thorough dissection of host-pathogen interactions, particularly demonstrated in cases of *M. marinum*, *M. avium* and *L. pneumophila* studies (King and Insall 2009). Pathogenic mycobacteria, such as *M. tuberculosis* or *M. marinum*, are able to survive and spread within *D. discoideum* cells with the characteristics of infection resembling macrophages (discussed in the *Mycobacterium marinum* section of introduction) (Fig.1.12). Besides, *Mycobacteria* and *Legionella*, studies have also been performed with other pathogens, for example it was shown that the autophagy pathway is required for resistance to *S. typhimurium* (Jia, Thomas et al. 2009).

![Figure 1.12. Electron microscopy of mycobacterial infection of macrophages and *D. discoideum*](image)

Figure 1.12. Electron microscopy of mycobacterial infection of macrophages and *D. discoideum* (A) *M. tuberculosis* infection of a human macrophage (Russel, 2002). Bacteria-containing electron-transparent and electron-opaque compartments may indicate diverse nature of the compartments. Another explanation may include the presence of cytosolic mycobacteria since bacteria-surrounding membranes for electron-opaque areas are not visible. (B) *M. marinum* infection of *D. discoideum*, electron microscopy by Monica Hagedorn.

*D. discoideum* was extensively used as a model system to study *Legionella* infection. Host models for *L. pneumophila* infection include guinea pigs, different protozoa, monocytes and other
types of human cells, while *D. discoideum* came in use much later (Hagele, Kohler et al. 2000, Solomon and Isberg 2000). Overall infection in *D. discoideum* with *L. pneumophila* appear to be similar to macrophages.

It was observed that uptake of *L. pneumophila* by *D. discoideum* occurs by means of macropinocytosis (Peracino, Balest et al. 2010), whereas in macrophages both macropinocytosis and phagocytosis appear to be involved (Watarai, Derre et al. 2001). Other differences include the speed of infection development inside the cells. In *D. discoideum*, the infection process occurs more slowly than in macrophages with the cell lysis taking place 48 hours post infection (Solomon and Isberg 2000, Lu and Clarke 2005).

In *D. discoideum* it was shown that depletion of mitochondria in the cells results in an increase in the *L. pneumophila* replication rate. Inhibition of AMP-activated protein kinase (AMPK), the central energy sensor, reversed that trend. On the contrary, overexpression of the AMPK catalytic subunit results in enhancement of intracellular growth of bacteria. Notably, AMPK is upregulated during intracellular infection of *L. pneumophila* but the exact role of AMPK in infection remains unclear. Another mitochondrial study revealed that *L. pneumophila* participates in disruption of mitochondrial protein synthesis during the course of infection of *D. discoideum*, particularly, decreases in mitochondrial mRNA levels as early as 4 hours post infection and cleavage of the large subunit of the mitochondrial rRNA were observed (Zhang and Kuspa 2009).

Host-pathogen interaction research can also utilize another interesting aspect of *D. discoideum*: its ability to alternate between unicellular and multicellular stages, the latter including slug and fruiting body formation (Fig.1.13). The migrating slug phase is particularly interesting due to the presence of the so-called Sentinel (S) cells that exhibit immune-like phagocytosis activity (Chen, Zhuchenko et al. 2007). The presence of immune-like function may potentially increase the chances of discovery of activities that are valid at the multicellular level.
1.5.2 Acanthamoeba castellanii as a host model for infection

Acanthamoeba castellanii is another amoebozoan host model system to study host-pathogen interactions. It is particularly interesting since Acanthamoeba faces Mycobacterium in its natural environment. Multiple Mycobacterium species have been shown to be phagocytosed and to be taken up by amoebal trophozoites into vacuoles, including *M. tuberculosis* (Mba Medie, Ben Salah et al. 2011), *M. bovis* and BCG strains (Taylor, Ahonen et al. 2003), *M. leprae* (Lahiri and Krahenbuhl 2008), *M. marinum* (Cirillo, Falkow et al. 1997), *M. avium* (Steinert, Birkness et al. 1998), *M. avium* subsp. Paratuberculosis (Mura, Bull et al. 2006) (Whan, Grant et al. 2006), *Mycobacterium kansasii* (Goy, Thomas et al. 2007), *M. xenopi* (Drancourt, Adekambi et al. 2007), *Mycobacterium fortuitum* (Cirillo, Falkow et al. 1997), *M. smegmatis* (Cirillo, Falkow et al. 1997), (Sharbati-Tehrani, Stephan et al. 2005), and 26 additional non-tuberculous species (Adekambi, Ben Salah et al. 2006).

Active interaction of *A. castellanii* with *Mycobacterium* species could indicate significant functional similarity of *Acanthamoeba* and macrophage responses to mycobacterial
infection. Moreover, comparative genomic studies report that the most recent common ancestor of *Mycobacterium* was an environmental bacterium (Ahmed, Saini et al. 2007, Stinear, Seemann et al. 2008) that evolved towards a variety of species specialized in living in different environmental niches including intracellular parasites such as *Mycobacterium avium*, *Mycobacterium tuberculosis* complex species, and *Mycobacterium leprae*.

Although *A. castellanii* does not provide the genetic tools of *D. discoideum*, whose genome is fully sequenced and annotated (Eichinger, Pachebat et al. 2005), it has the advantage of survival and growth at temperature above 25°C, in the temperature range for normal interactions between pathogens and human hosts, and *M. tuberculosis* with macrophages in particular (Goy, Thomas et al. 2007), a characteristic that makes *A. castellanii* an acceptable host system for infection. *A. castellanii* has served as a model organism to study interactions with bacteria including phagocytosis (Allen and Dawidowicz 1990), cell-surface receptors (Allen and Dawidowicz 1990), bactericidal mechanisms (Essig, Heinemann et al. 1997); this model facilitates the study of cytoskeletal and mitochondrial organization (Sluse and Jarmuszkiewicz 2002).

![Electron microscopy of *Acanthamoeba* sp.](image)

**Fig. 1.14** Electron microscopy of *Acanthamoeba* sp. (A) Transmission electron microscopy of a trophozoite. (B) Scanning electron microscopy of a cyst. (C) Transmission electron microscopy of a cyst. Ec, ectocyst; En, endocyst; M, mitochondria; N, nucleus; Op, operculum; Os, ostiole. Scale bars = 2 μm. Fuque, 2012
Mycobacterial infection of macrophages and *A. castellanii* have many similarities. For example, it was shown that *M. avium* mutants with a deletion of pathogenicity island genes displayed highly similar defects in infections of *A. castellanii* and human macrophages (Danelishvili, Wu et al. 2007). In *A. castellanii* an upregulation of 20 genes were found, similar upregulation was observed in macrophages. Discovered genes were involved in metabolic pathways, transcription and translation, and macromolecule degradation (Tenant and Bermudez 2006). The importance of mycobacterial outer membrane permeability in intracellular survival was investigated using porins *mspA* and *mspC* mutants that display enhanced persistence (Sharbati-Tehrani, Stephan et al. 2005). Deletion of *mspA* and *mspC* does not result in complete impermeability of the membrane indicating that *mspA* and *mspC* are not the only factors in membrane permeability. Overall studies suggest that amoebal trophozoites like *A. castellanii* may serve as a training field for mycobacterial species that enables survival within human macrophages by avoiding phagosome maturation.

A substantial amount of research was performed on the *Legionella* model of infection that also displayed high levels of similarity with *Legionella* infection of macrophages (Fig. 1.15). Like pathogenic mycobacteria, *L. pneumophila* is able to hijack phagosomes, preventing their fusion with lysosomes and forming specific ‘*Legionella*-containing vacuoles’ (LCVs) (Bozue and Johnson 1996). *L. pneumophila* displays cytotoxic effect on *A. castellanii* (Hagele, Hacker et al. 1998) and *A. polyphaga* (Gao and Kwaik 2000), with the cell death displaying characteristics of necrosis.

The *Acanthamoeba* model was used for the characterization of *Legionella* virulence factors, including the Icm/Dot (intracellular multiplication/defective organelle transport) T4SS (Segal, Feldman et al. 2005) (reviewed by Segal et al., 2005). Icm/Dot T4SS is a conjugation apparatus that injects more than 30 proteins into the host that was shown to be present both in protozoa and mammalian cells. The Icm/Dot T4SS mediates phagocytosis, inhibition of endocytosis, interactions with early secretory pathway components and release from the host. Although the Icm/Dot T4SS is essential for entry and host manipulation, it is not required for intracellular replication of *L. pneumophila*.

During *Acanthamoeba* infection, accumulation of PI(4)P occurs on the *Legionella*-containing vacuoles. PI(4)P is bound by the Icm/Dot-secreted protein SidC. LCVs also obtain properties of ER membranes, such as accumulation of calnexin (Bozue and Johnson (1996). Formation of the LCV is facilitated by the activities of the small GTPases Rab1, Arf1, and Sar1 and guanine nucleotide exchange factors (reviewed in (Hilbi, Weber et al. 2007)).
Figure. 1.15. Legionella infection of A. castellanii. (1) phagocytosis, (2) inhibition of endocytosis, (3) interaction with the early secretory pathway, (4) Replication of L. pneumophila in LCV and (5) release from amoebae A. The Icm/Dot T4SS of L. pneumophila governs phagocytosis, inhibition of endocytosis, interaction with the early secretory pathway and release from amoebae. Legionella-containing vacuoles accumulate PI(4)P, which is bound by the Icm/Dot-secreted effector protein SidC, and acquire ER markers such as calnexin. Formation of the LCV depends on the activity of the small GTPases Sar1, Arf1 and Rab1 as well as on Icm/Dot-secreted guanine nucleotide exchange factors. Replication of L. pneumophila in LCV does not seem to require the Icm/Dot T4SS (Hilbi, Weber et al. 2007).

1.5.3 Mammalian cell-based assays for drug discovery and host-pathogen interaction studies.

Working with protozoan models, one must take into account significant genetic difference between protozoan organisms and human cells. Therefore, usage of mammalian cell-lines for validation is necessary. Mammalian cell-based assays are suitable for distinguishing agonists and antagonists, identification of allosteric modulators and obtaining direct information on compounds about cell permeability and intracellular stability, and compound-associated cytotoxicity (Kunapuli, Lee et al. 2006). Moreover, cell-based assays provide experiments performed in a more biologically relevant microenvironment and therefore represent a compromise between the whole organism and in vitro systems. Unlike protozoan models, mammalian cell lines bear genomes more similar to humans, which is especially important in infectious diseases research. Furthermore, mammalian cell lines can provide responses that are tissue specific, and have been successfully used for primary screening, lead identification and optimization, and
screening for cytotoxicity. On the other hand, generation of mammalian cell lines could be more time-consuming due to diploid genome, which could provide limitations in mechanism of action studies. Mammalian cell-lines are also difficult to cultivate in the laboratory.

The rapid progress in combinatorial chemistry, genomics, proteomics, and bioinformatics has led to a significant increase in the number of potential therapeutics, which has also spurred the development of high-throughput screening (HTS) for lead identification and optimization. Over the past two decades, HTS has emerged and become one of the bases of drug discovery in the pharmaceutical industry.

Lead identification and optimization using cell-based assays is a growing trend in drug discovery. This approach provides higher biological relevance comparing to the standard biochemical assays. Big pharma and biotechnology companies are currently in the process of replacing other in vitro and biochemical assays with cell-based assays in the field of drug discovery. Currently, cell-based assays represent roughly half of all high throughput screens (Zhang and Yang 2011).

Cell-based HTS brought a number of positive results. For example, thrombopoietin receptor agonist Eltrombopag (Promacta/Revolade; GlaxoSmithKline) is a successfully discovered lead that was approved by The Food and Drug Administration (FDA) in 2008 (Macarron, Banks et al. 2011). Eltrombopag was selected out of a set of 260,000 compounds with the screen based on the use of luciferase reporters. The basis of the screen involved the thrombopoietin-responsive cell line, BAF-3/TPO-Rlic, genetically designed by transfecting murine hematopoietic progenitor cells (BAF-3) with a human TPO receptor (hTPOr) cDNA and a luciferase-expressing gene (Duffy, Darcy et al. 2001). TPO regulated a STAT-responsive promoter that controlled luciferase expression. Among other screened compounds, Eltrombopag showed high level of BAF-3/TPO-Rlic cell line proliferation and the increase of the amount of CD41+ cells (Duffy, Shaw et al. 2002, Erickson-Miller, DeLorme et al. 2005, Erickson-Miller, Delorme et al. 2009).

Mammalian cell-based screening also facilitated the discovery of BMS-790052 hepatitis C virus (HCV) NS5A (Bristol-Myers Squibb). It was selected as a clinical candidate that is able to inhibit hepatitis C virus proliferation (Macarron, Banks et al. 2011). This system measures replication of HCV replions, luciferase activity and cytotoxicity in a mixture of HCV and bovine viral diarrhea virus (BVDV) cell lines isolated from human hepatocarcinoma Huh-7 (O'Boyle, Nower et al. 2005). Alamar blue dye is used as a marker of cytotoxicity, while HCV replication is measured by the level of NS3 protease activity.

Finally, mammalian cell-lines such as cultured macrophages are extensively contributing to TB drug screening. For example, a screen of 57,000 small molecules led to identification of dinitrobenzamide derivatives with anti-mycobacterial activity (Christophe, Jackson et al. 2009). Compounds were active through inhibition of decaprenyl-phosphoribose 2’ epimerase DprE1/DprE2 and displayed anti-mycobacterial activity against M. tuberculosis, including
extensively drug resistant strains. Inhibition of decaprenyl-phosphoribose 2’ epimerase DprE1/DprE2 results in inhibition of formation of lipoarabinomannan and arabinogalactan, which are the main components of the mycobacterial cell wall.
2. Materials and methods
## 2.1. Materials

### 2.1.1 Media and buffers

<table>
<thead>
<tr>
<th>Medium and buffers</th>
<th>HL5 Medium including Glucose supplemented with vitamins and micro-elements pH 6.20 (Formedium)</th>
<th>HL5 (Cosson) medium pH 6.65</th>
<th>Low fluorescence medium (LoFlo)</th>
<th>Soerensen buffer pH 6.0</th>
<th>Phosphate buffer saline (PBS) pH 7.4</th>
<th>Middlebrook 7H9 (Difco)</th>
<th>DNA running buffer Tris acetate EDTA buffer (TAE) pH 8.0</th>
<th>6x DNA loading buffer Ethidium bromide</th>
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<tr>
<td></td>
<td>pH 6.20</td>
<td>pH 6.65</td>
<td>5.0 g/l Casein Peptone</td>
<td>5.0 g/l Yeast Extract</td>
<td>140 mM NaCl</td>
<td>4.7 g/ 900 ml</td>
<td>40 mM Tris-acetate</td>
<td>30 % Glycerol</td>
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<td></td>
<td>(Formedium)</td>
<td></td>
<td>0.35 g/l Yeast Extract</td>
<td>7.15 g/l Yeast extract</td>
<td>3.4 mM KCl</td>
<td>Add before autoclaving: 2 % glycerol 0.05 % Tween 80</td>
<td>1 mM EDTA</td>
<td>0.25 % Bromophenol blue in water or TE</td>
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<td>11.0 g/l Glucose</td>
<td>18.0 g/l Maltose monohydrate</td>
<td>1.8 mM KH2PO4</td>
<td>Add after cooling down to 50°C 10 % OADC</td>
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<td>SIH (Formedium)</td>
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<td>300 mg/l Cys</td>
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<tr>
<th>Dulbecco’s Modified Eagle Medium (Gibco)</th>
<th>Vitamins</th>
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<tr>
<td>30 mg/l Glycine</td>
<td>4 mg/l Choline chloride</td>
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<tr>
<td>84 mg/l L-Arginine hydrochloride</td>
<td>4 mg/l D-Calcium pantothenate</td>
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<tr>
<td>63 mg/l L-Cystine 2HCl</td>
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<td>580 mg/l L-Glutamine</td>
<td>4 mg/l Niacinamide</td>
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<tr>
<td>42 mg/l L-Histidine hydrochloride-H2O</td>
<td>4 mg/l Pyridoxine hydrochloride</td>
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<td>105 mg/l L-Isoleucine</td>
<td>0.4 mg/l Riboflavin</td>
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<td>105 mg/l L-Leucine</td>
<td>4 mg/l Thiamine hydrochloride</td>
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<td>146 mg/l L-Lysine hydrochloride</td>
<td>7.2 mg/l i-Inositol</td>
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<td>30 mg/l L-Methionine</td>
<td>Inorganic Salts</td>
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<tr>
<td>66 mg/l L-Phenylalanine</td>
<td>264 mg/l Calcium Chloride (CaCl2-2H2O)</td>
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<tr>
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<td>95 mg/l L-Threonine</td>
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<td>16 mg/l L-Tryptophan</td>
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</tr>
<tr>
<td>72 mg/l L-Tyrosine</td>
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</tr>
<tr>
<td>94 mg/l L-Valine</td>
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0.1 mg/l Ferric Nitrate (Fe(NO₃)₃·9H₂O)
200 mg/l Magnesium Sulfate (MgSO₄·7H₂O)
400 mg/l Potassium Chloride (KCl)
3700 mg/l Sodium Bicarbonate (NaHCO₃)
6400 mg/l Sodium Chloride (NaCl)
141 mg/l Sodium Phosphate monobasic (NaH₂PO₄·2H₂O)

Other Components
4500 mg/l D-Glucose (Dextrose)
15 mg/l Phenol Red
110 mg/l Sodium Pyruvate

PYG medium
20 g/l Proteose Peptone (BD 211684)
1.0 g/l Yeast extract
98.59 mg/l MgSO₄·7H₂O
5.92 mg/l CaCl₂·2H₂O
217.7 mg/l Sodium citrate·2H₂O
20 mg/l Fe(NH₄)₂(SO₄)₂·6H₂O 20mg
355 mg/l Na₂HPO₄·7H₂O
340 mg/l KH₂PO₄
18 g/l Glucose

2.1.2 Antibiotics

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<tr>
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<th>Working concentration</th>
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<tr>
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<td>10 U/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 mg/ml</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>100 mg/ml</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Hygromycin</td>
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<td>50 µg/ml</td>
</tr>
<tr>
<td>G418</td>
<td>10 mg/ml</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>Amikacin</td>
<td>20 mM/ml</td>
<td>10 µM/ml</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>30 mM/ml</td>
<td>10 µM/ml</td>
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2.1.3 Bacteria and cell cultures
Cell lines

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<thead>
<tr>
<th>Name</th>
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<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AX2</td>
<td>Dr. G. Gerisch</td>
<td></td>
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<tr>
<td>AX2 GFP-ABD</td>
<td>TS lab</td>
<td></td>
</tr>
<tr>
<td><em>A. castellanii</em> (ATCC 30234)</td>
<td>Dr. H. Hilbi</td>
<td>Tiaden, 2007</td>
</tr>
<tr>
<td>BV2 cells</td>
<td>M. Hagedorn</td>
<td>Lund et al., 2005</td>
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</table>

*Mycobacterium marinum* cell lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Received from</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium marinum</em> wt GFP</td>
<td>TS lab</td>
<td>(Hagedorn and Soldati, 2007)</td>
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<td><em>Mycobacterium marinum</em> wt Ds-red</td>
<td>TS lab</td>
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2.2 Methods

2.2.1 *D. discoideum* cultivation

2.2.1.1 Cell culture

*D. discoideum* cell lines are grown at 22°C, in HL-5c medium supplemented with 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco). Cells in adherent conditions are grown in Falcon Petri dishes (BD Biosciences) and split every 2-3 day before reaching confluency.

Cells in suspension are grown in conical flasks shaken at 0.5 g at a density of $10^5$-$10^7$ cells/ml with a cell density range between $5 \times 10^5$ and $5 \times 10^6$ cells/ml. Mutant cells lines are constantly kept under selection by addition of antibiotics.

2.2.1.2 Cell stocks

Cells from one confluent 10 cm dish are collected and counted using a Neubauer counting chamber. They are resuspended at a density of $5 \times 10^6$ cells/mL in HL5c 10% DMSO. Cells are divided into aliquots of 1 mL and immediately placed in ice-cold Nalgene MisterFreeze boxes filled with isopropanol. They are then transferred at -80°C for freezing. After 24h frozen cells are transferred in liquid nitrogen for long-term storage.
2.2.1.3 Spores

Cells are collected from subconfluent dishes. They are washed in Sorensen Buffer and resuspended at $5 \times 10^7$ cells/mL in Sorensen Buffer. 2 mL of the cell suspension are deposited on starvation agar plates (Sorensen buffer with 2% bacto-agar) and spread by swirling. The plates are incubated 30 minutes with an open lid until they are almost dry but still humid. They are then incubated upside down at 22°C for at least 24h in a humid atmosphere.

The sori, containing the spores, are collected in the lid of the plates by repeated tapping of the plate against the lid. They are vigorously resuspended in Sorensen buffer. After counting, they are washed in Sorensen Buffer and pelleted at 1200g, 5 min). They are then resuspended in Sorensen buffer + 10% glycerol at a density of $10^7$ spores/mL. 1mL aliquots are prepared and frozen in Nalgene MisterFreeze boxes. After 24h, they are transferred in liquid nitrogen for long-term storage.

2.2.2 D. discoideum infection

2.2.2.1 Preparation of the host cells

*D. discoideum* cells are cultivated at least overnight in medium without antibiotics, in adherent or in shaking conditions in sterile filtered HL5c. Cells grown in shaking condition are plated in 10 cm dish ($5 \times 10^7$ cells/dish) at least 30 min before infection. After adhesion, replace medium with 5 mL of fresh HL5c without antibiotics.

2.2.2.3 Preparation of the mycobacteria

Mycobacteria are grown to OD$_{600}$=0.8-1 in 7H9 (OD$_{600}$=1 corresponds to $5 \times 10^8$ bacteria/mL). Before infection, mycobacteria are transferred to 50 ml falcon tube and centrifuged at 800g for 10 seconds to pellet the bacterial aggregates to be discarded. Time of the centrifugation step may vary slightly depending on the exact size of the mycobacteria. Size of the pellet depends on the amount of aggregates in initial stage. Centrifugation does not sediment single bacteria. In case of low OD after centrifugation, resuspend the bacteria and repeat centrifugation for shorter time interval. Note that, for the same OD, the centrifuged culture contains more single bacteria than the syringed culture since it has no clumps at all. Centrifugation is also recommended before starting new shaking culture. Centrifugation step prevents forming of new bacterial aggregates for long time. For one infection, $5 \times 10^8$ mycobacteria (MOI=10) are washed twice in HL5c.

2.2.2.4 Infection

- Add $5 \times 10^8$ mycobacteria per plate of host cells
- Centrifuge at 500 g at RT for 2 x 10 min (in between rounds, gently move the dish to redistribute bacteria and turn the dish 180°)
- Let cells phagocytose for an additional 10-20 minutes
- Wash off extracellular bacteria with repeated rinses using filtered HL5c without antibiotics (2-5 washes)
- Bang the dishes horizontally and take up the cells in filtered VL5c + amikacin (final 10 μM amikacin) at 10^6 cells/ml density.
- Transfer 100 μL (10^5 cells) per well in 96-well plate (Cell Carrier, black, transparent bottom from Perkin-Elmer).
- Wait 20 min for cells to adhere.
- The infection status is monitored with a plate reader (Synergy Mx, Biotek) measuring fluorescence. Increased fluorescent signal displays the growth of the GFP-expressing mycobacteria inside D. discoideum cells. The plate reader temperature is pre-set at 25°C. Infection is monitored during 72 hours with the measurements were taken every 3-12 hours.

2.2.3 A. castellanii cultivation

A. castellanii are grown in adherent conditions at 25°C in T-75 tissue culture flasks (Milian), in approximately 8 ml of PYG medium or in 10 cm dishes in a medium without. Cells are split every 2-3 day before reaching confluency.

To split cells:
- Remove old media
- Add about 5ml of fresh PYG
- Bang the flask/dish against hard surface several times to detach the amoeba
- Dilute as required, topping up with PYG

One T-75 at near confluency will provide > 10 cm plates

A. castellanii should be grown in a 25°C incubator, although they can survive temperatures of up to 37°C. Acanthamoeba is much less sensitive to overgrowth than D. discoideum, reaching 100% confluency is still acceptable as long as the cells are still attached to the plate.

2.2.3.1 A. castellanii cell stocks

Cells from one confluent 10 cm dish are collected and counted using a Neubauer counting chamber. They are resuspended at a density of 10^6 cells/mL in PYG plus 10% DMSO. Cells are divided into aliquots of 1 mL and immediately placed in ice-cold Nalgene MisterFreeze boxes filled with isopropanol. They are then transferred at -80°C for freezing. Frozen cells are transferred in liquid nitrogen for long-term storage after 24h.

2.2.4 A. castellanii infection

2.2.4.1 Preparation of the host cells

Acanthamoeba castellani cells are cultivated as usual in PYG medium without antibiotics, in adherent conditions. Cells grown in shaking condition are plated in 10 cm dish (2 x 10^6 cells/dish) at least 30 min before infection. After adhesion, replace medium with 5 mL of fresh PYG without antibiotics.
2.2.4.2 Preparation of the mycobacteria

Mycobacteria are grown to OD$_{600}$=0.8-1 in 7H9 (OD$_{600}$=1 corresponds to $5 \times 10^8$ bacteria/mL). Before infection mycobacteria are transferred to 50 ml falcon tube and centrifuged at 800 g for 10 seconds to pellet and discard bacterial aggregates. Time of the centrifugation step may vary slightly depending on the exact size of the mycobacteria. Size of the pellet depends on the amount of aggregates in initial stage. Centrifugation does not sediment single bacteria. In case of low OD after centrifugation, resuspend the bacteria and repeat centrifugation for shorter time interval. Note that, for the same OD, the centrifuged culture contains more single bacteria than the syringed culture since it has no clumps at all. Centrifugation is also recommended before starting new shaking culture. Centrifugation step prevents forming of new bacterial aggregates for a long time. For one infection, $5 \times 10^8$ mycobacteria (MOI=10) are washed twice in PYG and centrifuged at 800 g for 10 seconds to discard the aggregates.

2.2.4.3 Infection

- Add $1 \times 10^5$ mycobacteria per well of host cells
- Centrifuge at 500 g at RT for 2 x 12 min (in between rounds, gently move the dish to redistribute bacteria and turn the dish 180°)
- Let cells phagocytose for an additional 10-20 minutes
- Wash off extracellular bacteria with repeated rinses using PYG without antibiotics (2-5 washes)
- Bang the dishes and take up the cells in 5 mL PYG + amikacin (10 μM final concentration). Amikacin is added to prevent extracellular growth of mycobacteria.
- Plate on the 96-well plate (Cell Carrier, black, transparent bottom from Perkin-Elmer).
Figure 2.1. The scheme of *A. castellanii* infection for compounds screening. GFP-expressing *M. marinum* is centrifuged on the top of monolayer of *A. castellanii*. Extracellular bacteria are washed away. Afterwards, compounds and amikacin are added (10 μM final concentration of amikacin). Monitoring of the infection is performed with the use of Synergy H1 or MX fluorescence plate reader. Measurements are taken every 3-12 hours for 60-72 hours.

2.2.4.4 Monitoring the infection

Plate reader assay Infections with fluorescent *M. marinum* are monitored with a plate reader (Synergy Mx, Biotek) measuring fluorescence. Increased fluorescent signal monitors growth of the mycobacteria inside *A. castellanii* cells. An example of the data, obtained by fluorescent measurements is presented in Fig. 2.2.

The plate reader temperature is pre-set at 32°C.

2.2.5 BV2 cells cultivation

Murine microglial BV2 cells are grown at 37°C in an incubator with 5% CO₂, in DMEM medium supplemented with fetal bovine serum (10% final) without antibiotics to grow in adherent conditions to reach 50-70% confluency the day of the experiment. Cells in adherent conditions are grown in Falcon Petri dishes (BD Biosciences) and split every 2-3 day before reaching confluency. To split the cells discard the medium, wash 1 time with 10 ml PBS buffer and add 1 ml 1x Trypsin-EDTA and let the plate for 5 minutes at 37°C. Resuspend the cells in 10 ml of DMEM, split 1:10. It is very important not to let BV2 reach 100% confluency since the cells start to die quickly. While working with BV2 cells, gloves must be used all the time.

2.2.6 BV2 cells infection

BV2 cells are cultured in DMEM medium supplemented with FCS in 10 cm Petri dishes at 37°C. The day prior to infection, cells are trypsinized, passaged and transferred to 96-well plates
(black transparent bottom from BD Falcon), so as to reach a 60-70% confluency the day of the experiment. Note that BV2 cells do not grow significantly during the infection.

*M. marinum* are cultured in a shaking culture at 32°C up to an OD600 of 0.8–1 in 7H9 medium supplemented with OADC as described before. Mycobacteria are centrifuged onto pre-plated BV2 cells at an MOI of 2-5 to promote efficient and synchronous uptake. Centrifugation is performed at RT at 500 g for 10 min in a clinical centrifuge. After an additional 20 min incubation (important not to keep BV2 cell exposed to bacteria for longer time since this is cytotoxic), extracellular bacteria are washed off with DMEM and infected cells are resuspended in DMEM supplemented with FCS. Compounds are added to a 30 µM final concentration. Infected cells are incubated at 32°C. The course of infection is monitored by high-content microscopy with time points taken every 7-14 hours.

### 2.2.7 High-content microscopy

Cells are monitored in 96-well plates (black, transparent bottom from BD Falcon). Recording of transmitted light and GFP fluorescence data is performed using ImageXpress Micro XL Widefield High content Screening System (20x 0.75 NA, air).

### 2.2.7.1 Fluorescence Microscopy

*A. castellanii* or BV2 cells are infected with GFP-expressing *M. marinum* as described above. Infected cells are monitored in 96-well plates (Cell Carrier, black, transparent bottom from Perkin-Elmer).

Recordings are performed using a Leica LF6000LX microscope (40x 1.4 NA air) or High-Content microscopy. Quantification of the image fluorescence intensity is used to determine the effect of compounds versus vehicle controls. The activities of the compounds are determined by analysis of the maximum difference of fluorescence intensity at day 3 post infection compared to 6-12 vehicle controls.
2.2.7.2 Phase Contrast Microscopy

Acanthamoeba, Dictyostelium and BV2 cells infected with mycobacteria are monitored in vivo by phase contrast microscopy infected with GFP-expressing M. marinum. Infected cells are monitored in 96-well plates (Cell Carrier, black, transparent bottom from Perkin-Elmer). Recordings
are performed using a CKX41 inverted microscope. Phenotype of infection is observed, including efficiency of infection, number of intracellular and extracellular bacteria, growth rates of *M. marinum* and BV2 cells. Cytotoxic effects, such as rounding of cells, detachment from the surface of the well and debris production is taken into account. Three points are taken for monitoring the infection: one in the center of the well and two at the periphery.

![Table showing experiment results](image)

**Figure 2.5. Example of High-Content phase contrast microscopy for cytotoxicity assay.** BV2 cells at $10^5$ initial cell density are presented. An overview of 96-well plate is presented.

![Image showing microscopy results](image)

**Figure 2.6. Example of the phase contrast microscopy of a single well**

A. *A. castellanii* are monitored at 60 hours post incubation. B) Growth inhibition
2.2.8 Growth Inhibition Assay

$10^5$ GFP-expressing *M. marinum* are transferred to each well of 96-well plates. Bacterial growth at 25°C is monitored by measuring the GFP fluorescence in a fluorescent platereader (Synergy H1) for 72 hours with a time point taken every 3 hours.

2.2.9 Z-factor calculation

The Z factor is calculated using the means and standard deviations of both positive and negative controls (mp, sp and mn, sn). The following formula is applied: $Z\text{-factor} = 1 – 3(sp+ sn)/|mp - mn|$

![Figure 2.7. Example of Z-factor calculation](image)

Figure 2.7. Example of Z-factor calculation. $10^5$ BV2 cells are transferred into 96-well plate. In 48 wells 10 μM rifabutin was added. In other 48 plates not contain compounds. Z-factor is calculated based on the values of the last time points of cumulative curves. Y-axis represents total fluorescent intensity (first time point subtracted), X-axis represents hours post infection. Mycobacteria do not display growth in the wells with inclusion of rifabutin (flat lines).

2.2.10 Spectrum analysis

Spectrum analysis is performed with the use of the Synergy MX fluorescent plate reader. Parameters of excitation and emission wavelength are altered in order to find and determine peaks of maximum excitation and emission.
Figure 2.8. Example of excitation and emission spectrums for *M. marinum* expressing Ds-red.

Various colors of spectrum profiles represent different excitation values, X axis indicates emission values. Emission spectrum for *M. marinum* expressing the Ds-red reporter, in 7H9 medium.
3. Results
3.1 Quantitative light-based measurements for drug screening

The choice of equipment and parameter settings are crucial in any drug screening. In many cases, the equipment determines the type of signal measured, the dynamic range of the measurements, the sensitivity, the reproducibility, the signal-to-noise ratio, and the speed and thus overall duration of the screening. For our drug screening we selected the Synergy MX and Synergy M H1 plate readers that allow measurement of three types of signals: fluorescence intensity, luminescence intensity and absorbance. The Synergy plate readers are useful for drug screenings due to their ability to make use of 6- to 384-well plates. Combination with additional equipment such as the Twister II microplate handler allows automated readings of up to 80 plates per round of infection, which renders the whole screening procedure robust and high-throughput. Naturally, a preliminary exploration of the various plate reader parameters is important to establish the capabilities and limits of the screening system. During assay establishment, we tested all possible modes of reading.

3.1.1 Selection of parameters for the fluorescence plate reader

**Endpoint measurements.** The endpoint mode is the fastest type of fluorescent reading with a read speed of 11 seconds per 96-well plate, but its usage has limitations. The reading is performed in the center of the well in an area of around 2000 microns in diameter. The diameter of the entire area of the well is around 6604 microns. This means that the measurement is performed for only 11% of the well surface. Such an approach is only appropriate if the signal is homogeneously distributed in the whole well (Fig. 1A). When the signal is not homogeneous, different areas of the well may result in different values. For example, fluorescent bacteria or cells in suspension may form clumps that may move across the surface (Fig. 1B). This may often occur in complex *in vivo* systems such as an infection. Endpoint kinetics mode is a very effective way of reading in the absence of clumps and other heterogeneities. It is especially useful for the recording of fast changes of the system with the best ability to produce kinetic curves with reads every 11 seconds per 96-well plate.

![Figure 3.1. Variations of endpoint RFU measurements depending on homogeneity of the system.](image)

A. From $10^5$ to $10^6$ GFP-expressing *M. marinum* were transferred to the 96-well plate in 7H9 broth. Total...
fluorescence intensity were monitored for 2 hours (kinetics mode). B. More than $10^8$ GFP-expressing *M. marinum* were transferred to the 96-well plate in 7H9 broth. Total fluorescence intensity was measured for 4 hours. Short period of measurements (2-4 hours) allows to minimize the effect growth and, instead, observe changes in fluorescence distribution.

**Area scan mode.** Unlike the end point measurements, the area scan mode is capable of performing multiple reads in one well. It produces maximum coverage of the well area, including the center and the periphery, and generates statistically robust data within one well, including standard deviation and mean value. Since our growth and infection assays display some level of heterogeneity, the area scanning mode was used. The parameters of the area scan were selected as presented in Table 3.1.

![Figure 3.2. Area scan mode selection (fluorescence).](image)

There are multiple ways to select reading areas in the well. We decided to use 3x3 mode with inclusion of all points. Slight overlap of the reading with the wall of the well didn’t significantly affect the measurements. Increasing the number of points results in significant increase in the overall time of measurements (Fig.3.2).

**Plate type.** There are several different plates to consider. Fluorescence emitted from cell monolayers is measured more reliably in black plates with a transparent bottom, since they minimize background noise. Luminescence is measured more efficiently in white plates, because reflection of the light allows for collection of the complete luminescence signal.

**Number of wells.** The Synergy MX and H1 plate readers facilitate readings in the range from 6 to 384-well plates. 96-well plates were considered to be optimal for our type of system. The use of higher volumes allows more precise reading and minimizes factors such as pipetting error.
**Bottom and top modes of reading.** There are two ways to perform the measurement: either from the top or from the bottom. We selected bottom reading because cells and bacteria are concentrated mostly at the bottom of the well. Overall, in our hands top and bottom modes of reading did not differ significantly.

**Excitation and emission.** Excitation and emission parameters are determined by the fluorescent properties of the fluorophores used. In our case these include GFP, DS-red, and mCherry reporters. Optimal parameters of peaks of excitation and emission may vary slightly depending on the bandwidth value that determines the ‘window’ of wavelength in which the detection is performed.

**Spectrum.** Spectra measurements are used to identify profiles of excitation and emission of the sample experimentally. Knowledge of emission profiles allows selection of fluorescent reporters with minimal interference with background emission of the medium.

**Table 3.1. Selected area scan options (fluorescence):**

<table>
<thead>
<tr>
<th>Read method</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Read type</td>
<td>area scanning</td>
</tr>
<tr>
<td>Optics type</td>
<td>Monochromators</td>
</tr>
<tr>
<td>Read step parameters</td>
<td></td>
</tr>
<tr>
<td>Excitation:</td>
<td>485 GFP</td>
</tr>
<tr>
<td>Emission:</td>
<td>515 GFP</td>
</tr>
<tr>
<td>Optic position:</td>
<td>Bottom</td>
</tr>
<tr>
<td>Gain</td>
<td>85</td>
</tr>
<tr>
<td>Read speed:</td>
<td>Normal</td>
</tr>
<tr>
<td>Read height:</td>
<td>8 mm</td>
</tr>
<tr>
<td>Measurements per data point:</td>
<td>6</td>
</tr>
</tbody>
</table>

**Area scan options**

| Number of points             | 3 x 3 (Horizontal x Vertical) |
| Point Spacing (microns)      | 1648 x 1648                  |
| Well Diameter                | 6604 microns                 |
| Probe Diameter               | 2000 microns                 |

**Gain (sensitivity).** The gain is another important characteristic of the reading. The gain determines the range of fluorescent intensities that can be measured at the same time. Too high values of gain result in signal overflow. For our purpose, the sensitivity was set in the range of 70 to 100. Changing of the gains results in increase of voltage to the photomultiplier tube, which in turn makes it more sensitive.
The fluorescence intensity was measured for various cells and media at various gain values. The relationship between gain and RFU seems to behave exponentially (Fig. 3) which could be expected since photomultiplier tube multiply electrons exponentially due to the process of secondary emission. The exact empirical relationship for calculating new values of RFU with the gain change is described by the following formula:

\[ \text{RFU}_2 = \text{RFU}_1 \times (\text{Gain}_2/\text{Gain}_1) \]

Where

- \( \text{RFU}_2 \) – new RFU data with the new gain
- \( \text{RFU}_1 \) – RFU data of the same well with the old gain parameters
- \( \text{Gain}_2 \) – new gain parameters
- \( \text{Gain}_1 \) – old gain parameters

Using this formula, samples measured at different gains are comparable by live microscopy.

**Figure 3.3. Relationship between RFU data and gain (sensitivity) values.** \( 2 \times 10^5 \) *D. discoideum* cells expressing a green fluorescent protein (GFP) fusion with the actin-binding domain of filamin (GFP-ABD) were transferred to the well of 96-well plate in HL5c medium and in 10%HL5c plus 90% Soerensen sorbitol buffer. Total fluorescence intensities were measured at different gain values. Media without cells was used in controls.
3.1.2 Plate reader absorbance parameters selection

**Absorbance.** The absorbance is another mode of reading. It reflects the overall turbidity of the sample. Like the fluorescent signal, the absorbance signal can be performed in two modes: area scanning and end point measurements with similar defining characteristics. End point kinetics were used primarily for homogeneous solutions. The heterogeneous infection assay was monitored by area scanning. The parameters of the area scan were selected as presented below.

After various rounds of testing, the following parameters were selected for absorbance measurements (Table 3.2).

Table 3.2. Absorbance measurements selected:

<table>
<thead>
<tr>
<th>Read method</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Read type</td>
<td>area scanning</td>
</tr>
<tr>
<td>Optics type</td>
<td>Monochromators</td>
</tr>
<tr>
<td>Read step parameters</td>
<td></td>
</tr>
<tr>
<td>Wavelength</td>
<td>600</td>
</tr>
<tr>
<td>Delay after plate movement</td>
<td>100 msec</td>
</tr>
<tr>
<td>Gain</td>
<td>85</td>
</tr>
<tr>
<td>Read speed:</td>
<td>Normal</td>
</tr>
<tr>
<td>Measurements per data point:</td>
<td>8</td>
</tr>
<tr>
<td>Area scan options</td>
<td></td>
</tr>
<tr>
<td>Number of points</td>
<td>3x3 (Horizontal x Vertical)</td>
</tr>
<tr>
<td>Point Spacing (microns)</td>
<td>1648 x 1648</td>
</tr>
<tr>
<td>Well Diameter</td>
<td>6604 microns</td>
</tr>
<tr>
<td>Probe Diameter</td>
<td>1400 microns</td>
</tr>
</tbody>
</table>

Notably, the diameter of the absorbance area scan is different from the fluorescence intensity area scan (Fig. 3.4). It is 1400 microns in diameter. This means that values of fluorescence intensity and absorbance values cannot be compared directly.

**Absorbance spectrum.** The spectrum mode facilitates the monitoring of variations of absorbance values depending on excitation. Although the standard excitation wavelength is 600 nm for bacteria and cells, we did not observe significant changes in samples behavior at different excitation wavelengths. No phototoxic activities were observed even though absorbance values rise significantly for shorter wavelengths. This occurs due to fact that the amount of refraction increases as the wavelength of light decreases because shorter wavelengths of light are slowed more and consequently experience more bending than do the longer wavelengths. Apparently, the excitation time is too short for cytotoxicity induction.
Figure 3.4. Area scan mode selection (absorbance). Big circles represent the area of the well in a 96-well plate. Blue circles represent the probe size where absorbance signal is measured. Dotted lines mark the probes that are not taken into account during mean fluorescent intensity calculation. 3x3 area scan mode (corners included) mode was selected for the screen.

Figure 3.5. Absorbance at different excitation values. Absorbance was measured for HL5c medium at different excitation values (from 300 nm to 700 nm). No cells were added.

Total time-length of the read. The total time-length of readings was monitored in order to select the optimal mode of reading for medium-throughput screenings. There are several parameters
that affect the total reading time: the number of probes, the number of reads per probe, the delay setting, the energy parameters, and the number of wells. The delay setting sets the delay after plate movement in the plate reader to prevent the effect of vibration on readings. In our case, delay did not result in significant changes of measurements. The energy mode that defines the energy of excitation also did not affect the readings. Overall differences are summarized in the Table 3.3.

**Low and high energy modes.** These two modes determines which light source would be used during reading. Low energy mode corresponds to tungsten lamp while high energy corresponds to xenon flash. Tungsten lamp provides strong, stable light output in visible range, although, it has less sensitivity and no light output below 300 nm. On the other hand, xenon flash provides higher energy and slightly higher sensitivity than tungsten lamp, although it is more expensive.

Table 3.3. Time-length of the reads in various settings.

<table>
<thead>
<tr>
<th>Read method</th>
<th>Number of reads</th>
<th>Number of probes</th>
<th>Energy mode</th>
<th>delay</th>
<th>Time of the reading of the plate</th>
<th>Number of wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>area scan</td>
<td>6 reads</td>
<td>5x5</td>
<td>high energy</td>
<td>no delay</td>
<td>9min59s</td>
<td>96</td>
</tr>
<tr>
<td>area scan</td>
<td>6 reads</td>
<td>5x5</td>
<td>low energy</td>
<td>no delay</td>
<td>8min17s</td>
<td>96</td>
</tr>
<tr>
<td>area scan</td>
<td>6 reads</td>
<td>3x3</td>
<td>high energy</td>
<td>no delay</td>
<td>5min08s</td>
<td>96</td>
</tr>
<tr>
<td>area scan</td>
<td>6 reads</td>
<td>3x3</td>
<td>low energy</td>
<td>no delay</td>
<td>3min44s</td>
<td>96</td>
</tr>
<tr>
<td>endpoint</td>
<td>6 reads</td>
<td>1</td>
<td>high energy</td>
<td>no delay</td>
<td>45s</td>
<td>96</td>
</tr>
<tr>
<td>endpoint</td>
<td>6 reads</td>
<td>1</td>
<td>low energy</td>
<td>no delay</td>
<td>33s</td>
<td>96</td>
</tr>
<tr>
<td>endpoint</td>
<td>10 reads</td>
<td>1</td>
<td>low energy</td>
<td>no delay</td>
<td>33s</td>
<td>96</td>
</tr>
<tr>
<td>endpoint</td>
<td>10 reads</td>
<td>1</td>
<td>high energy</td>
<td>no delay</td>
<td>52s</td>
<td>96</td>
</tr>
<tr>
<td>endpoint</td>
<td>10 reads</td>
<td>1</td>
<td>high energy</td>
<td>100msec</td>
<td>3min10s</td>
<td>384</td>
</tr>
<tr>
<td>endpoint</td>
<td>10 reads</td>
<td>1</td>
<td>high energy</td>
<td>no delay</td>
<td>2min32s</td>
<td>384</td>
</tr>
<tr>
<td>endpoint</td>
<td>10 reads</td>
<td>1</td>
<td>low energy</td>
<td>no delay</td>
<td>1min58s</td>
<td>384</td>
</tr>
<tr>
<td>OD</td>
<td>8 reads</td>
<td>1</td>
<td>100msec</td>
<td>3min11</td>
<td>384</td>
<td></td>
</tr>
<tr>
<td>OD</td>
<td>8 reads</td>
<td>1</td>
<td>no delay</td>
<td>2min33</td>
<td>384</td>
<td></td>
</tr>
</tbody>
</table>
3.1.3 Fluorescence spectrum analysis

Spectrum analysis was performed to investigate possible interference with the signal by background fluorescence of the media. Excitation and emission were tested in the range of 300 to 700 nm with an increment of 3 nm. We found that the Loflo medium showed the lowest fluorescent intensity peak while HL5c and PYG demonstrated a high level of autofluorescence (Fig. 3.6).

Figure 3.6. Emission spectra of the media. Various colors of spectrum profiles represent different excitation values, X axis indicates excitation values. A. Spectrum profiles of Loflo. B. Spectrum profiles of HL5c. C. Spectrum profiles of PYG medium.

Spectrum analysis of fluorescent reporters. Results of the spectral analysis indicate acceptable level of background fluorescence for signal measurements of fluorescent reporters. Peaks of excitation and emission corresponds to published data of the fluorescent reporters (GFP, mCherry, Ds-red).
Fluorescence spectra were measured for model organisms as well (Fig. 3.7).

**Figure 3.7. Emission spectra of the model organisms.** Various colors of spectrum profiles represent different excitation values, X axis indicates emission values. A. Emission spectrum for *M. marinum* expressing mCherry reporter, in 7H9 medium. B. Emission spectrum for *M. marinum* expressing the Ds-red reporter, in 7H9 medium. C. Emission spectrum of *D. discoideum* expressing the GFP reporter, in HL5c medium.

### 3.1.4 Fluorescence growth assays

Bactericidal activity and cytotoxic activity are crucial characteristics of drug-like compounds. Both parameters are usually measured in traditional screening approaches. In order to measure these activities, we established an assay that measures the fluorescence intensity and the OD as indicators of the number of mycobacteria or cells. Fluorescence measurements allow us to estimate the growth of bacteria/number of cells. The inhibition of bacterial growth indicates either bactericidal of bacteriostatic activity, while the absence of changes indicates a lack of activity affecting growth. Notably, in our assay we normally did not observe a decrease of GFP fluorescence intensities lower than initial fluorescent intensity due to low degradation rates of GFP. Due to the stability of GFP bacterial killing does not always result in decrease of fluorescence intensity which renders the assay suboptimal for detection of bactericidal activities. Further decrease of the intensity could be facilitated.
by prolongation of total duration of measurements, or by usage of a secondary luminescence-based assay. The latter assay allows us to discriminate between growth inhibition and actual bactericidal activity.

In our screen, both bacterial and cell growth assays are performed over a 3 days period. This duration is significantly shorter than a typical screening in broth with slow growing bacteria such as *M. tuberculosis* or BCG, allowing us to increase the throughput of the assay. Potential drawbacks may include the overlooking of slow acting anti-mycobacterial drugs such as pyrazinamide. On the other hand, fast growth rates of *M. marinum* might enhance the bactericidal activity of some drug candidates. Our assays are based on pathogenic *M. marinum* and *D. discoideum* as for growth inhibiton and/or-cytotoxicity studies.

### 3.1.4.1 *M. marinum* growth assay

This *M. marinum*-based assay resembles standard antibiotic assays performed with BCG and *M. tuberculosis* except that a measurement of total fluorescence of constitutively expressed fluorescent reporters (GFP) substitutes for the CFU counting. Additionally, as mentioned before, the duration of the measurements is shorter due to the faster growth rates of *M. marinum*

During assay establishment, various parameters have to be taken into account. Firstly, it is important to be sure that total fluorescence intensity is proportional to the number of cells. Indeed, serial dilutions of *M. marinum* expressing GFP display direct proportionality to the measured fluorescent intensities (Fig. 3.8). Microscopy observations also show stable fluorescent expression in 100% of bacteria.

![Figure 3.8. Correlation between fluorescence intensity and serial dilution.](image)

Serial dilutions of GFP-expressing *M. marinum* were transferred to 96-well plate. Dilution factor is 3. A relative density unit corresponds to $10^5$ bacteria.

We use numbers of bacteria that are similar to the ones used in the infection assay. Similar bacterial loads allow us to adequately compare the activities of compounds *in vitro* and *in vivo*. Therefore, we used $10^5$ bacteria per well as an initial number for the antibiotic assay.
*M. marinum* displays a classical mycobacterial growth dynamics with exponential phase and slowing down of the growth at high densities. Observation of the plateau phase is not possible due to saturation of the signal. Monitoring of such high numbers of bacteria is not necessary since they are not biologically relevant. Nevertheless, lack of observations at high densities may result in overlooking some effectors of metabolic changes related to high bacterial load. However, the majority of the hits can still be detected at lower densities. Potent anti-mycobacterial drug candidates are capable of decreasing the bacterial load starting from small quantities of bacteria.

**Figure 3.9. M. marinum growth in 7H9.** $10^5$ GFP-expressing *M. marinum* were transferred to the well of 96-well of 96-well plate, 7H9 broth. Fluorescent intensity was measured. Measurements were performed every 3 hours for 72 hours.

The antibiotic assay was performed in 96-well black plates with a transparent bottom as described in the protocol section. Before starting the measurements $10^5$ *M. marinum* were transferred to each well. Monitoring was performed in 200 µl of the 7H9 broth. Observations were performed every 3-12 hours over 60-72 hours.

In order to validate our assay, we used a set of different compounds with known bactericidal activities. We successfully tracked bactericidal activities that reduced the rates of total fluorescence increase (Fig. 3.10, 3.11, 3.12, 3.13). For example, rifampicin, a first line TB drug, displayed bactericidal activity starting from 30 nM. Amikacin also displayed strong growth inhibition *in vitro*.

At the same time we did not observe significant activity for the majority of compounds with no published anti-mycobacterial activity (Fig. 3.13). This indicates that our assay is highly specific for anti-mycobacterial activities.
Figure 3.10. Rifampicin effect on *M. marinum* growth. $5 \times 10^5$ *M. marinum* were transferred to the well, in 7H9 broth. Rifampicin was added at indicated concentrations. Fluorescence intensities were measured every 3 hours for 72 hours. Where fluorescence intensity was saturated these data were excluded from analysis.

Figure 3.11. Fusidic acid effect on *M. marinum* growth. $5 \times 10^5$ *M. marinum* were transferred to the wells, in 7H9 broth. Fusidic acid was added at indicated concentrations. Fluorescence intensities were measured every 3 hours for 72 hours. Where fluorescence intensity was saturated these data were excluded from analysis.
Figure 3.12. Streptomycin effect on *M. marinum* growth. 5×10⁵ *M. marinum* were transferred to the wells, in 7H9 broth. Streptomycin was added at indicated concentrations. Fluorescence intensities were measured every 3 hours for 72 hours. Where fluorescence intensity was saturated these data were excluded from analysis.

Figure 3.13. 1H-Indole-5-carboxamide effect on *M. marinum* growth. 5×10⁵ *M. marinum* were transferred to the wells, in 7H9 broth. 1H-Indole-5-carboxamide was added at indicated concentrations. Fluorescence intensities were measured every 3 hours for 72 hours. Where fluorescence intensity was saturated these data were excluded from analysis.

We tested most standard anti-tubercular compounds, and the majority of them showed MIC values that are comparable to the ones obtained in assays using *M. tuberculosis* (data not shown). IC50
values of the anti-mycobacterial drugs tested are displayed in the Table 3.4. The antibacterial activities were also confirmed by high-content microscopy (described in section 2.2.7).

Table 3.4. Inhibitory concentrations 50% (IC50) values for *M. marinum* growth in extracellular conditions (Kicka, Trofimov et al. 2014).

<table>
<thead>
<tr>
<th>antibiotic</th>
<th>extracellular IC50 (μM)</th>
<th>intracellular IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>streptomycin</td>
<td>0.1–1</td>
<td>3–7</td>
</tr>
<tr>
<td>fusidic acid</td>
<td>0.01–0.1</td>
<td>3–5</td>
</tr>
<tr>
<td>ethionamide</td>
<td>0.1–1</td>
<td>3–10</td>
</tr>
<tr>
<td>isoniazid</td>
<td>5–10</td>
<td>20–30</td>
</tr>
<tr>
<td>rifampin</td>
<td>0.1–0.5</td>
<td>3–10</td>
</tr>
<tr>
<td>rifabutin</td>
<td>2–3</td>
<td>1–3</td>
</tr>
<tr>
<td>ethambutol</td>
<td>5–10</td>
<td>5–10</td>
</tr>
<tr>
<td>levofloxacin</td>
<td>10–30</td>
<td>&gt;30</td>
</tr>
<tr>
<td>moxifloxacin</td>
<td>5–10</td>
<td>&gt;30</td>
</tr>
<tr>
<td>thioridazine</td>
<td>1–5</td>
<td>5–10</td>
</tr>
<tr>
<td>PA-824</td>
<td>3–5</td>
<td>5–10</td>
</tr>
</tbody>
</table>

3.1.4.2 *D. discoideum* growth assay.

Cytotoxicity is an extremely important characteristic of the compounds. Biosafety is an obligatory requirement of drug-like compounds. We selected a method to measure cytotoxicity by quantifying cell growth. Inhibition of cell growth indicates possible cytotoxic activity. Distinguishing between growth inhibition and cytotoxicity was performed by microscopy analysis.

*D. discoideum* cells expressing a green fluorescent protein (GFP) fusion with the actin-binding domain of filamin (GFP-ABD) display stable fluorescence. Cell dilutions are proportional to cell numbers. Characteristics of the growth curve suggest that *D. discoideum* stops growing soon after reaching 100% confluence in the well, which is indicated by the plateau phase of the growth curve (Fig. 3.14). The doubling time of 8–9 hours fits observations reported in the literature.
Figures 3.14. *D. discoideum* growth in HL5c. $10^5$ GFP-ABD-expressing *D. discoideum* cells was added to the well in HL5c medium. Fold increase of fluorescence intensity was calculated (background subtracted). Measurements were performed every 3 hours for 52 hours.

To achieve more physiologically relevant results, it is important to estimate an optimal cell density, when the signal is high enough for precise measurements. A quantity of $10^5$ provided the highest fold-increase for 72 hours measurements (Fig. 3.15).

Figure 3.15. Effect of *D. discoideum* cell density on cell growth. GFP-ABD-expressing *D. discoideum* cell densities in a range up to $9 \times 10^5$ are presented. Measurements were performed in HL5c medium every 3 hours during 44 hours.

Various parameters were tested such as the components of the final mixture. DMSO is an example of such a component. Knowing its effect on cell growth is crucial since it is a vehicle
compound used in screening. DMSO displays cytotoxic activity at high concentrations; at low concentrations in our screen no significant growth retardation activity was observed. This means that the use of DMSO as a compound solvent is acceptable up to 0.5% concentration (Fig. 3.16).

**Figure 3.16. DMSO effect on growth of GFP-ABD-expressing *D. discoideum*.** $10^5$ *D. discoideum* cells were measured in HL5c medium plus different concentrations of DMSO. Fold increase of fluorescence intensity was calculated (background subtracted). Measurements were performed for 48 hours every 3 hours.

We also considered SIH and Loflo as alternatives to the HL5c medium. An advantage of SIH is that it is chemically defined. On the other hand, Loflo has significantly lower autofluorescence. Our observations show that *D. discoideum* survives less efficiently in SIH; cells start dying after reaching confluence as indicated by a drop in fluorescence (Fig. 3.17). Nevertheless, use of SIH for growth and infection assays might still be possible if optimal cell densities are chosen.
Figure 3.17. GFP-ABD-expressing *D. discoideum* growth in SIH medium at various cell densities. *D. discoideum* cells were added at indicated cell densities per well. Fluorescence intensities were measured every 3 hours for 56 hours.

Loflo demonstrates very low levels of growth which was expected, because it is a low nutrient medium (Fig. 3.18). Such a high level of inhibition makes it useless for long-term measurements.

![Graph](image1.png)

Figure 3.17. GFP-ABD-expressing *D. discoideum* growth in SIH medium at various cell densities. *D. discoideum* cells were added at indicated cell densities per well. Fluorescence intensities were measured every 3 hours for 56 hours.

We hypothesized that SIH medium lacks some specific micro-nutrient that is present in HL5c. Titrations of SIH mixtures with HL5c were tested in order to compensate for this discrepancy (Fig. 3.19). Characteristics of the curves indicate that *D. discoideum* growth is inhibited in SIH even with

![Graph](image2.png)

Figure 3.18. *D. discoideum* GFP-ABD growth in Loflo medium at various cell densities. GFP-ABD *D. discoideum* cells were added at cell densities indicated. Fluorescence intensities were measured every 3 hours for 56 hours.
25% addition of HL5c. A seemingly linear growth of the cells in SIH mixtures is most likely caused by a prolonged lag phase.

![Figure 3.19. GFP-ABD-expressing D. discoideum growth, titration of SIH and HL5c. Percentage of HL5c are indicated. $10^5$ GFP-ABD D. discoideum cells were added to the well with SIH plus titrations of HL5c medium (percentages are indicated).](image)

Other cytotoxic compounds were tested (Fig. 3.20, 3.21). This finding shows that we can use this assay in cytotoxicity / cell growth inhibition detection. Overall, the majority of the tested compounds did not display cytotoxic or growth inhibition activities.

![Figure 3.20. Effect of gentamycin on GFP-ABD-expressing D. discoideum growth. $10^5$ D. discoideum cells were transferred to the wells, in HL5c medium. Gentamycin was added at indicated concentrations. Fluorescence intensities were measured for 66 hours every 3 hours.](image)
Figure 3.21. Effect of caffeine on GFP-ABD-expressing *D. discoideum*. $10^5$ *D. discoideum* were transferred to the wells, in HL5c medium. Caffeine was added at indicated concentrations. Fluorescence intensities were measured for 66 hours every 3 hours.

### 3.1.5 Absorbance assays

Absorbance measurements are a possible alternative to fluorescence or luminescence measurements. Unlike fluorescence assays, the absorbance assay does not require genetic manipulations or selective markers. Moreover, it is easy to perform with the use of Synergy MX and H1 plate readers. However, absorbance measurement have drawbacks such as low accuracy for the monitoring of heterogeneous solutions. Low cell numbers are also problematic due to the high transparency of amoebae.

We established growth assays both for *A. castellanii* and *D. discoideum*. During assay development, we observed linear correlation at high numbers of amoebae. Changes in excitation parameters influence the background, but the overall absorbance pattern stayed constant (Fig. 3.22). At the same time, we did not observe any phototoxic activity. Use of *D. discoideum* expressing GFP-ABD allowed us to compare fluorescence with absorbance measurements in parallel. Although an increase of absorbance signal was observed (Fig. 3.25), the pattern was different from total fluorescence intensities (Fig. 3.23). The *A. castellanii* growth assay demonstrated high robustness for lower cell numbers. This fact indicates that *A. castellanii* is less transparent than *D. discoideum*.

The absorbance is also affected by other factors. For example, condensation in the lid can significantly affect the measurements and change optical characteristics of the well. We performed a trial screening with the use of BV2 cells and GFP-expressing *M. marinum* (Fig.3.24). Due to relatively
inconsistent results of the trial screening we decided to choose total fluorescence over absorbance measurements as a more robust measurement.

Absorbance measurements could be an acceptable choice in an assay with strict humidity control that prevents condensation. The use of absorbance measurements could be more problematic in more complex heterogeneous systems, such as infection assays. For infection assays, the absorbance represents an ambiguous measurement, since it is not clear how optical characteristics of both host and pathogen change during infection. Measurements of bacteria in broth also may not provide optimal results due to optical changes of single bacteria and bacterial clumps (Fig. 3.26).

![Figure 3.22. Correlation between absorbance and serial dilutions of A. castellanii. A. castellanii were transferred to 96-well plate at various cell densities in PYG medium. 1 relative A. castellanii absorbance unit corresponds to 10^3 A. castellanii.](image)

![Figure 3.23. Comparison of GFP-ABD-expressing D. discoideum fluorescence intensities values and absorbance measurements. A. D. discoideum growth measured by fluorescence intensity (cell density per well is indicated). B. D. discoideum growth measured by absorbance for the same wells.](image)
Figure 3.24. BV2 infection assay with absorbance measurements. Example of infection assay screening based on absorbance measurements. $5 \times 10^4$ infected cells were transferred to the wells. Time points were taken every 6-24 hours. The plate was incubated at 37°C in the presence of 5% CO$_2$.

Figure 3.25. *D. discoideum* AX2 growth measured by absorbance values. $10^5$ cells were used as initial cell density. Measurements were performed at 25°C for 44 hours.
Figure 3.26. Corellation between absorbance measurements and serial dilutions of *M. marinum*.

1 relative *M. marinum* density unit corresponds to $3.2 \times 10^5$ bacteria.

### 3.1.6 Infection assays

Detection of anti-infective activities is more relevant for the situation in an infected whole organism than antibacterial activities since it takes into account *in vivo* criteria, such as cell membrane permeability, intracellular degradation, molecular pumps and others. *In vivo* assays tend to be oriented towards drug-likeness since any antibacterial drug has to display activity *in vivo*.

Therefore, our aim was to establish infection assays that allow monitoring of antibacterial conditions in physiologically relevant environments. As a host system for primary screening we decided to use *A. castellanii*. *A. castellanii* is easy to work with, it is large, and is an efficient phagocyte that can survive at 37°C, the temperature of standard human infection. Besides *A. castellanii* assays, additional optimization experiments were performed in *D. discoideum* expressing GFP-ABD in order to take advantage of genetic manipulation for further studies on the mode of action of the identified compounds.

Ideally, an infection assay must specifically monitor the intracellular growth of the bacteria. However, this is quite difficult to achieve, since bacteria are able to escape the host via cell death, exocytosis or ejection. In this case, researchers may detect extracellular activities of the compounds that are similar to activities in broth. Extracellular growth can also mask intracellular changes. Monitoring of non-stop intracellular growth during 72 hours seems to be a challenging problem.

In order to deal with this challenge, three approaches can be applied. The first one includes use of a very low MOI, resulting in quick uptake of the extracellular bacteria. Another approach involves multiple rounds of washes to eliminate non-ingested bacteria. Both of these methods tend to increase the error in screenings, either due to a decrease in the signal level or a possible loss of bacteria and cells during the washing step.
The third approach is based on the use of antibiotics that kill extracellular bacteria while letting intracellular bacteria proliferate. This approach allows an initial increase in bacterial load and to avoid additional interventions during 3 days. Amikacin is a great example of such a useful compound. It has a high activity on extracellular bacteria and a very low one on intracellular bacteria, as shown in macrophages. However, there are some drawbacks. Some of the screened compounds may have synergistic effects with amikacin or target amikacin-related pathways resulting in false-positive hits. These pseudo-hits can be easily eliminated by establishing additional assays for primary hit validation that test synergistic activities of the compounds.

### 3.1.6.1 A. castellanii infection assay

We established an assay based on infection of A. castellanii with M. marinum, expressing GFP with a monitoring of the infection for 60-72 hours by fluorescence plate reader Synergy MX and Synergy H1. One reader is in our lab, the other is part of the Access platform of the NCCR Chemical Biology. Both readers were used in order to maximize throughput of the screening. We did not observe significant differences between normalized fluorescence data derived from both plate readers. Detection was performed in PYG medium containing 10 µM amikacin to prevent extracellular bacterial growth.

Indeed, we observed no increase of fluorescence in broth (Fig. 3.27, Fig. 3.28A, B); while in an infected culture of cells, the fluorescence was still increasing (Fig. 3.29). This allows us to suggest that we observe intracellular growth of the bacteria.

![Figure 3.27. Amikacin effect on M. marinum in 7H9 broth.](image) Fluorescence intensities of M. marinum (background subtracted), initial M. marinum density is 10⁵ bacteria per well. Measurements were performed for 72 hours every 3 hours.
**Figure 3.28. Amikacin effect on M. marinum in 7H9 medium 72h broth.** A. Negative control after 72 hours in 7H9. B. M. marinum in the presence of 10 µM amikacin after 72 hours.

**Intracellular growth of M. marinum**

We observed that intracellular growth of *M. marinum* differs from its growth in broth (Fig. 3.29). Indeed, intracellularly, *M. marinum* multiplies in a relatively linear fashion, unlike its exponential growth in broth. This may reflect overall slowing down of metabolic rates inside the host that is also observed for *M. tuberculosis* or combination of two types of events: bacterial replication and death. The intracellular effect of amikacin might also be a factor.

**Figure 3.29. Intracellular growth of M. marinum in A. castellanii infection.** Linearity of fluorescence increase is observed.
**Cell density effect on infection**

The multiplicity of infection is important in experiments with infected *A. castellanii*. Mycobacterial growth rates are affected by starting cell density. Observations show the highest change of mycobacteria load when the starting density was $5 \times 10^4$ cells per well. We took this number as an initial cell number for infection in order to maximize overall precision of the measurements.

![Figure 3.30. *A. castellanii* cell density effect on *M. marinum* infection. Total fluorescence intensity was measured over time. Experiments were performed in triplicate. (Kicka, 2014).](image)

Another important characteristic is MOI of infection. It is important to maintain a high percentage of infected cells, yet to minimize the number of extracellular bacteria remaining after the washing steps. Our optimization experiments showed that with an MOI higher than 10, the percentage of infected cells stays at a similar level (Fig. 3.30).

Therefore, we decided to use MOI 10. At MOI 10 our assay results in a low percentage of extracellular bacteria, which was confirmed by fluorescence microscopy (Fig. 3.31).
3.1.6.4 SIH *D. discoideum* assay

Unlike HL5c, SIH has the benefits of being a chemically defined medium. It also has slightly lower autofluorescence than HL5c medium. Therefore, we decided to make an SIH medium-based infection, similar to the HL5c infection but with a change of the medium prior to transfer of the cells to 96-well plates. However, the use of SIH resulted in unexpected characteristics in mycobacterial growth curves.

The infection in the presence of SIH is characterized by rapid increase of GFP fluorescence intensity in the first 15-20 hours of infection, followed by a decline (Fig. 3.32). The apparent growth rate of *M. marinum* is higher than values reported in the literature. It may indicate possible technical reasons for fast fluorescence change like movement of bacteria into, or out of the optimal detection zone.

![Figure 3.32. *M. marinum* growth in *D. discoideum* infection in SIH medium. 5*10^4 infected cells were transferred to the wells.](image-url)
Microscopy analysis shows a fast release of the bacteria soon after the beginning of infection. After one day of infection a majority of bacteria are extracellular (Fig. 3.33). Increase of fluorescence most likely reflects reinfection of *D. discoideum*.

**Figure 3.33.** Release of intracellular *M. marinum* by *D. discoideum* in SIH medium. A. GFP-ABD-expressing *D. discoideum* (green) and mCherry-expressing *M. marinum* (red) in the beginning of infection. B. GFP-ABD-expressing *D. discoideum* and mCherry-expressing *M. marinum* (red) at 24 post infection hours.

### 3.1.6.3 *D. discoideum* infection assay in HL5c

*D. discoideum* is a good protozoan host candidate for *M. marinum* infection. Mutants expressing fluorescence reporters allow easy microscopy and quantification of the cells, which is important both for infection and microscopy assays (Fig. 3.34). However, infection of *D. discoideum* with *M. marinum* expressing GFP has its drawbacks. Detection is limited due to high autofluorescence of HL5c medium, which masks intracellular growth of *M. marinum*. There are two ways around this problem that include changing of either the fluorescent reporter or mode of measurement. We decided to keep the fluorescent reporter as an already established fluorescence system and change the mode of measurement to high-content microscopy.
3.1.6.4 BV2 infection assay

Infection of mammalian cell lines is an extremely important validation step in the screening of compounds with anti-mycobacterial activities. The BV2 cell line is a promising candidate since it is an immortalized macrophage-like microglial cell line that is easy to cultivate. BV2 cells display high level of infection and high phagocytic activity. BV2 grow at 37°C and standard physiological condition for human TB.

In order to standardize our conditions we made our BV2 assay as similar to the A. castellanii assay as possible. We also used 96-well plates, bottom read in the presence of 10 µM amikacin. The requirement for an atmosphere containing 5% CO\(_2\) represents a slight problem since our plate readers do not have CO\(_2\) regulation. In order to deal with this problem, we performed the incubation in a CO\(_2\) chamber, followed by plate sealing with a gas impermeable seal. M. marinum demonstrates linear-like intracellular growth similar to the A. castellanii model (Fig. 3.39). However, changes in DMEM color indicated a decrease in the CO\(_2\) levels in some of the wells. Lack of CO\(_2\) may interfere with the measurements and give false positive results. We decided to perform the monitoring in a high content microscope combined with a CO\(_2\) chamber.
3.2. Library selection and characterization

3.2.1 Sinergia library

The library of compounds was constructed by the group of Prof. Scapozza (Pharmacy department, University of Geneva) supported by a SNSF Sinergia grant. Compounds for each query were selected from ZINC database of 2.5 million compounds with the use of shape-based superposition method (Table 3.5). 25,000 compounds for each query were selected with the usage of Rapid Overlay of Chemical Structures (ROCS)-based virtual screening.

The ROCS requires a query as an input and a database of pharmacophores we would like to compare to the query (Table 3.5). The query represents the features of active ligands important for their high binding affinity against a certain target.

As a result of virtual screening ROCS produced a ranked list of ligands from the screened database. High ranked ligands were chosen for experimental studies. Compounds in this library target 18 known biochemical pathways both from pathogen and host sides, including amino acid metabolism, lipases, channels, ion transporters, fatty acids biosynthesis, catabolism of glucose and pyruvate, iron import/uptake, ATP synthases and others (Fig.). Structural similarity was calculated by Lingo method present in the OpenEye VIDA program. Finally about 100 compounds were selected for each pathway. Compounds were designed to cover the chemical and biological space related to ligands known to interact with bacterial virulence factors and host targets.

Figure 3.35. *M. marinum* growth in BV2 infection. Linearity of fluorescence increase is observed.
Figure 3.36. **Sinergia library design.** 1800 compounds were selected. Compounds were selected from ZINC database of 2.5 million compounds with the use of shape-based superposition method followed by TanimotoCombo score and Lingo methods with the usage of ROCS-OpenEye software. Each pathway includes a set of about 100 compounds in order to provide maximal chemical and biological space coverage.
Table 3.5. Example of the query for Sinergia library. Queries 1-4 build based on active ligands co-crystallized with the PI3K. Queries are represented with color atoms: green - ring, red - hydrogen bond acceptor, blue - hydrogen bond donor. At each color atom there is a weight x2, x3 which represents the importance of the corresponding physical property. The weight is user defined based on the available SAR and structural data. The shape of the queries is not shown due to the clarity reasons. EF1%, 10%, 20% - Enrichment factors after 1%, 10% or 20% of the dataset. Enrichment factors were calculated by a formula: EF=(HITSSampled /Nsampled)/(HITStotal/Ntotal). HITSSampled is number of experimentally found active structures in the top x% of the focused dataset, Nsampled is number of all structures in the top x% of the dataset, HITStotal is total number of active structures in the focused dataset and Ntotal is total number of all molecules in the focused dataset (Agata Kranjc Pietrucci and Leonardo Scapozza).

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Figure 3.37. Sinergia library biochemical pathways. Compounds in this library target 18 known biochemical pathways both from pathogen and host sides, including amino acid metabolism, lipases, channels, ion transporters, fatty acids biosynthesis, catabolism of glucose and pyruvate, iron import/uptake, ATP synthases and others. Each pathway includes a set of about 100 compounds in order to cover maximal chemical and biological space.

3.2.2 Malaria box

Malaria box is an open access library that includes 400 diverse compounds with antimalarial activity, including 200 diverse drug-like compounds as starting points for oral drug discovery and development. Drug-like compounds follow the rule-of-5-compliant physicochemical properties, such as absorption, distribution, metabolism and excretion (Lipinski 2004). However, their oral absorption has not been confirmed through in vitro or in vivo pharmacokinetic studies. Fulfilling rule-of-5 criteria makes drug-like compounds most useful as starting points for drug discovery activities such as lead optimization. Another 200 selected molecules are diverse probe-like compounds for use as biological tools in malaria research. Probe-like compounds do not possess rule-of-5-compliant physicochemical properties. They therefore might find most use as tools for probing biological mechanisms of action against a particular target. All these compounds have been screened in vitro against 3D7 (chloroquine (CQ) sensitive but sulfadoxine resistant strain of P. falciparum) (Duffy and Avery 2012). Cytotoxicity assays were performed on human embryonic kidney cell lines (HEK-293). Malaria parasites incubated with the compounds for 72 hrs were stained with DAPI in 384 wells.

Figure 3.38. Malaria box library design. 400 compounds in total were selected.
3.2.3 GSK TB-set

A subset of freely available GlaxoSmithKline library was selected from a collection of 2 million GSK compounds through the process of TanimotoCombo, software filtering and MIC generation. Compounds were tested in vitro on BCG and H37Rv strains. The selected set comprises 177 compounds that showed bactericidal activity against both mycobacterial strains.

![Diagram](image)

Figure 3.39. GSK TB-set hit library design. 177 compounds were selected.

3.3 Screening results

3.3.1 Screening results of Sinergia library of compounds

During A. castellanii infection screening, overall, 1260 compounds were screened in A. castellanii and M. marinum infection assay. Experiments were performed in quadruplicate. Identified primary included 22 hits with antiinfectious activity (1.7% hit rate), 44 hits with pro-infectious activity (3.5% hit rate). Cutoff of 2-3σ was used for primary hits selection. Cumulative curves of fluorescence intensity were generated as described in the manuscript and in Fig. 3.40 and Fig. 3.41. The summary of identified anti-infectives is presented in Table 3.6. Pro-infectives are presented in Table 3.7.
Figure. 3.40. Example of fluorescence cumulative curves of 9 vehicle controls in A. castellanii and M. marinum infection assay. \(5 \times 10^4\) infected A. castellanii cells were transferred to the wells, in DMEM medium. Fluorescence intensities were measured for 66 hours every 3 hours.

Figure. 3.41. Example of fluorescence cumulative curves of A. castellanii and M. marinum infection screen of the Sinergia library of compounds. \(5 \times 10^4\) infected A. castellanii cells were transferred to the wells, in DMEM medium. Sinergia library compounds were added at 30 μM. Fluorescence intensities were measured for 66 hours every 3 hours. Infections with compounds with no significant antimycobacterial activities show growth patterns similar to vehicle controls.

Table 3.6. Anti-infectives identified in primary A. castellanii and M. marinum infection screen. The fold increase is less than 0.8 in comparison to the vehicle control. Amount of variation indicated by sigma values.

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**Table 3.7. Pro-infectives identified in *A. castellanii* and *M. marinum* infection screen of the Sinergia library of compounds.** The fold increase is more than 1.3 in comparison to the vehicle control. Amount of variation is indicated by sigma values.
Highest hit rate for anti-infective compounds was for autophagy and methyl-citrate pathways, followed by tryptophan and AKT1 pathways. No primary hits were identified for FASII-PDIM metabolism, porins and lipases pathways (Fig. 3.4A).

For pro-infectious compounds, majority of the hits belonged to the cations channels category, ABC transporters and autophagy pathways (Fig. 3.4B).

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<th>CysPath</th>
<th>Autophagy</th>
<th>ABCTransporters</th>
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Figure 3.42. Distribution of the primary hits obtained in *A. castellanii* infection screen. A. Anti-infectious hits, 22 hits were obtained in total. B. Pro-infectious hits, 44 hits were obtained in total.

After initial screened, validation screening was performed. During the validation stage, compounds were screened again at 30 μM in duplicates in *A. castellanii* infection by GFP-expressing *M. marinum*. For that *A. castellanii* cells were infected with *M. marinum* Compounds were tested for
bactericidal/bacteriostatic activity in the growth assay of *M. marinum* expressing GFP. Cytotoxic properties of the hits were addressed with the use of growth assay of *D. discoideum* expressing GFP-ABD (Table 3.7). Changes of fluorescence intensities of *D. discoideum* expressing GFP-ABD were monitored in 96 well plates for 60-72 hours as described in

**Table 3.7. Final validation of anti-infectives identified in *A. castellanii* and *M. marinum* infection assay.** The normalized fluorescence intensities are indicated for *A. castellanii* infection assays, *M. marinum* growth assay and *D. discoideum* growth assay are indicated.

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Altogether, 6 compounds with strong *A. castellanii* inhibition, 4 compounds with antibiotic and anti-infectious activity, 6 pure anti-infectious compounds and 3 antibiotic activities were identified.

An infection assay with BV2 cells and *M. marinum* expressing GFP was used for generalizing the data activities of obtained *A. castellanii* infection screen. Interestingly, we were unable to reproduce pro-infectious activity of the compounds in BV2 cells. Apparently, discovered pro-infectious activities are either host-specific or assay-specific. Therefore, we decided to focus on anti-infectives. We validated 12 anti-infective compounds and SAR analogs among identified *A. castellanii* hits (Fig. 3.43). Among identified hits, 5 compounds displayed relatively strong level of *M. marinum* inhibition (more than 0.4-fold decrease). We decided to choose cutoff of 0.8-fold inhibition, subsequent more profound SAR studies will hopefully lead to identification of more potent SAR analogs.

**Figure 3.43.** Anti-infectious activities of primary hits in BV2 infection. BV2 cells were infected with GFP-expressing *M. marinum*. Infected cells at a transferred to the 96-well plate at initial density of 5×10⁴ cells per well. Y-axis corresponds to fold increase in comparison to the values of fluorescent intensity of the vehicle control at 60 hours post infection.
3.3.2 Screening results of Malaria box compounds

*A. castellanii* cells were infected with *M. marinum* expressing GFP in the presence of 5 µM of each compound. Intracellular bacterial growth was monitored by measuring the fluorescence intensity (Kicka et al, 2014). Results of a biological triplicate experiment are presented in Figure 3.45, one compound was found to possess clear anti-infective activity, with more than 50% inhibition of bacterial growth. In parallel, compounds were screened in *M. marinum* growth inhibition assay (Fig. 3.44) to identify possible antibiotic activities. *D. discoideum* growth inhibition assay was used for determination of growth inhibition and cytotoxic activities (Fig. 3.43).

As could have been expected, the efficiency of anti-malarials as anti-infectives in our mycobacterial infection model was very low. Some of the MMV’s compounds displayed slightly pro-infective activity, but this effect was mostly host-specific. At the same time, compounds didn’t display significant level of growth inhibition in *D. discoideum* assay. None of them totally prevented the growth of the amoeba, but some were cell-growth inhibitors with more than 50% inhibition (Table 1, Figure 3.43). Finally, a strong anti-infectious hit was identified (MMV000604) giving around 70% intracellular bacterial growth reduction. Interestingly, this compound does not have significant antibiotic activity and it does not have a cytotoxic or growth-inhibition effect on the host cells.

![Figure 3.43. Results of *D. discoideum* expressing GFP-ABD growth assay of Malaria box library.](image)

*D. discoideum* growth in HL5c. 10^5 GFP-ABD-expressing *D. discoideum* cells was added to the well in HL5c medium. Fold increase of fluorescence intensity after 60 hours was calculated (background subtracted). Y-axis represents fluorescence intensity normalized to mean value of 9-12 vehicle controls. Error bars represent the standard deviation of 3 biological replicas. Compounds are ordered from lowest to highest values per plate. Plate A contains both drug-like and probe-like compounds, plates B and C contain drug-like compound, plates D and E contain probe-like compounds.
Figure 3.44. Results of *M. marinum* expressing GFP growth assay of Malaria box compounds. 10^5 GFP-expressing *M. marinum* was added to the well in 7H9 medium. Fold increase of fluorescence intensity after 60 hours was calculated (background subtracted). Y-axis represents fluorescence intensity normalized to mean value of 9-12 vehicle controls. Error bars represent the standard deviation of 3 biological replicas. Compounds are ordered from lowest to highest values per plate. Plate A contains both drug-like and probe-like compounds, plates B and C contain drug-like compound, plates D and E contain probe-like compounds.

Figure 3.45. Results of *M. marinum* expressing GFP / *A. castellanii* infection assay of Malaria box compounds. Y-axis represents fold increase of total fluorescence intensity of control cumulative curves.
$5 \times 10^4$ *A. castellanii* cells infected with GFP-expressing *M. marinum* were added to the well in PYG medium. Fold increase of fluorescence intensity after 60 hours was calculated (background subtracted). Y-axis represents fluorescence intensity normalized to mean value of 9-12 vehicle controls. Error bars represent the standard deviation of 3 biological replicas. Compounds are ordered from lowest to highest values per plate. Plate A contains both drug-like and probe-like compounds, plates B and C contain drug-like compound, plates D and E contain probe-like compounds.
3.4 Chapters, papers and manuscripts.

**In vivo activities of compounds from the GSK TB-set tested in protozoan and mammalian model systems of mycobacteria infection**

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In preparation.

VT’s contribution: writing of the manuscript, Figures 1, 2, 3, data analysis, participated in development and optimization of fluorescence-based *A. castellanii*/GFP-expressing *M. marinum* infection assay, development of microglial BV2 infection assay, development of *M. marinum* antibiotic assay, cell cultivation.
In vivo activities of compounds from the GSK TB-set tested in protozoan and mammalian model systems of mycobacteria infection
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Summary:
Fighting the threat of tuberculosis requires combined yet diversified efforts of academic and industrial research groups and pharmaceutical companies. One of these efforts, initiated by GlaxoSmithKline in the form of a massive phenotypic screening, resulted in the identification of 177 compounds with highly potent antimycobacterial properties. The compounds were identified using M. bovis BCG and subsequently confirmed in M. tuberculosis H37Rv. Drug-like activities and cytotoxic properties of the compounds were also tested. However in vivo antimycobacterial activities remain to be tested, ideally in multiple model systems. Our research group joined the open-source, early-stage drug discovery effort and focused on the quantitation of antimycobacterial activities within three type of hosts infected with M. marinum. These model organisms include two species of free-living amoebae D. discoideum and A. castellanii plus the murine microglial BV2 cell line. The GSK TB-set of antituberculc compounds has been tested for cytotoxic, growth inhibitory and anti-infectious properties. As a pathogen, we used M. marinum a relatively fast growing and technically convenient bacterium that is an accepted model for the pathogenesis of M. tuberculosis.

Introduction.
Tuberculosis remains one of the most serious health-threat worldwide with the death toll exceeding 1.4 million people per year. The constant risk of emergence of new multiple resistance strains fuels the search for new chemical entities with antitubercular activities. The discovery of new drugs is a challenging issue due to unique properties of M. tuberculosis, particularly its low metabolic rates, highly impermeable cell wall and ability to manipulate the innate immune system via infection of alveolar macrophages. However, the generally high attrition rates of the recent pharmaceutical efforts forces us to rethink the strategies used in anti-infective drug discovery campaigns. It is getting clear that there is no obvious quick and easy solution for problems related to TB research. Such a situation favors diversification of
methodologies to facilitate the quest for effective drug-like compounds. The diversification includes further establishment of in vivo assays and expansion of the repertoire of model systems. Protozoan hosts are one of the possible alternative model systems. A. castellanii, a fresh water solitary amoeba and D. discoideum, a soil social amoeba possess, like macrophages, many of the conserved characteristics of professional phagocytes. At the same time amoebae are known for their ease of cultivation and manipulation. The haploid genome of D. discoideum provides expanded opportunities for fast and effective genetic engineering. On the other hand, M. marinum is a close relative of the M. tuberculosis complex that faces free-living amoebae in its natural habitat, thus representing a potentially powerful alternative Mycobacterium for drug screening in a variety of host-pathogen systems. Advantages of the M. marinum system include BSL2 level of biosafety combined with TB-like pathogenesis and relatively fast growth rate, particularly beneficial in screenings. In our work we decided to investigate intracellular activities of compounds from the GSK TB-set and explore potential advantages and limits of protozoan hosts and M. marinum in anti-TB drug development.

Results

Antibiotic activity against M. marinum

Compounds have been assayed at 10 µM directly against Mycobacterium marinum. According to the given MIC90 values [Ballell, 2013 #1], M. tuberculosis growth is greatly affected at this concentration. Interestingly, in our M. marinum antibiotic assay (see materials and methods [1]), 20 out of 168 compounds were found to have negligible effect (less than 20% inhibition) on bacterial growth (Table 1, appendix1, Figure 1A). These results might be explained by genetic and metabolic differences between M. marinum and M. tuberculosis or by technical reasons. For example, our assay is run over 3 days, and thus may potentially overlook compounds that require longer -time to exert full antimycobacterial effects.

Growth inhibitory activity on Amoeba

Prior to testing the TB-set compounds in our in vivo infection assays, a growth assay was performed using the amoeba Dictyostelium discoideum (Kicka et al, 2014) to evaluate the compounds’ toxicity. Results overall confirm that the vast majority of the compounds lack any cytotoxic activity, although some differences were also found. Three of the tested compounds showed complete growth inhibition of the amoeba and six inhibited cell growth by more than 50% (Table 1, Figure 1B). Surprisingly, even though the potential cytotoxicity of the compounds had been tested against various cultured animal cells (Ballel et al), strong
levels of inhibition was observed for 8 compounds using the murine microglial cell line BV2 (Appendix 5).

*A. castellanii - M. marinum infection assay*

We were particularly interested to test the *in vivo* activity during infection with *M. marinum*, in order to partially complement *M. bovis* BCG and *M. tuberculosis* H37Rv assays in broth. *A. castellanii* cells were infected with a GFP-expressing *M. marinum* strain in presence of 10 µM of each of the compounds. Intracellular bacterial growth was monitored by measuring the total fluorescence using a plate-reader (Kicka *et al.*, 2014; materials and methods [3]). Results of a biological triplicate experiment are presented in Table 1 and classical kinetic curves of intracellular bacteria from the three plates of compounds are presented in Figure 1C. Eight compounds (4.8 %) were found to possess significant anti-infective activity, with more than 50% inhibition of bacterial growth. Paradoxically, twenty-three compounds (13.7%) also demonstrated clear pro-infective activity, resulting in up to more than 30% increase of bacterial growth over the DMSO control. It must be emphasized that the best pro-infectives of these compounds (more than 70% increase), have a name that starts with the acronym “BRL-” in the GSK list, belong to the same chemical family (appendix 2) and exclusively build-up this pro-infective class.

**Half-inhibitory concentration determination and structure-activity relationship studies in an amoeba system**

In order to monitor the specificity and sensitivity of our amoeba infection model, we decided to experimentally determine the half-inhibitory concentration of the maximum anti-infective efficacy of related compounds. The imidazo[1,2-a]pyridine-3-carboxamide family was chosen for this study (appendix 3) since some of the members of this family displayed strong antiinfective activities in our BV2 and *A. castellanii* infection assays. These compounds were assayed at different concentrations in the *A. castellanii – M. marinum* infection model with at least two biological replicates for each concentration. The IC₅₀ of each compound was determined using the GaphPad Prism software. Finally, the compounds were sorted in four groups, depending on their potency.

**Microglial cell -M. marinum infection assay**
Moreover, a second assay was established to monitor quantitatively the infection in BV2 microglial cells. The assay was designed to be similar to \textit{A. castellanii} assay for the ease of results comparison. For this, BV2 cells were infected with GFP-expressing \textit{M. marinum} and intracellular bacterial growth was monitored for three days using time-lapse imaging with a high-content fluorescence microscope (figure 1). Analysis of raw images (total fluorescence measurement) was performed with the imageJ software. Results of a duplicate experiment are presented in Table 1, and microscopy pictures are presented in Figure 2. Seventeen drugs were found to exert significant anti-infective activity (10%), whereas twenty-one pro-infective compounds were identified with this BV2 model system.

**IC\textsubscript{50} determination in BV2 cells**

In order to further validate and expand the generality of the results obtained with the amoeba system, we determined the inhibitory concentrations of each compound during infection of murine microglial BV2 cells with \textit{M. marinum}. GSK TB-set were tested in concentrations ranging from 0.04 μM to 50 μM. The assay were designed similar to the one used for \textit{A. castellanii}. Based on these measurements, IC50 were calculated (Supplementary table 2). Compounds displayed an average IC\textsubscript{50} of around 6.5 with standard deviation of 3.9.

**Discussion**

As expected antibiotic that are efficient in an infection model is drastically reduced in both model, only 10% at max are efficient \textit{in vivo} in one of our model. Moreover, some antibiotics have been found to be pro-infectious when tested against infected cell in both model. Possible explanations may include the idea that reduce of mycobacterial fitness may actually be beneficial for bacteria by enhancing the survival of the host that in turns becomes more resourceful and efficient reservoir of infection. The model suggests that weak antibiotics can cause deacrease of the fitness of bacteria without killing them, which in turn cause increase of the host fitness. Fit hosts, in turn, benefit bacteria by providing resources and benefiting in spreading of infection. Indeed, in our \textit{A. castellanii} and BV2 infection assays we observed mostly intracellular proliferation of mycobacteria. It is true for BV2 infection assay without addition of amikacin, suggesting that \textit{M. marinum} prefers intracellular growth to extracellular. Illustration of the model is displayed in Figure 3.
Another explanation may include interference with amikacin in the medium. Antibiotic may weaken bacteria enough for host to survive and prevent exposition to extracellular amikacin.

This result is not fully understood so far but should lead to further studies to understand the underlying mechanism. Finally, few compounds have been found anti-infectious in amoeba model but 50% of them are also found in BV-2 model which seems less stringent. Because of its easiness and low-budget of cultivation, amoeba model appears to be a good alternative model before assaying compounds in a more complex system. Strong filters of the amoebic assays may help to get rid of false positive hits that mammalian assay provide, therefore benefiting the overall screening efficiency. However, A. castellanii host cells gave possibility to a massive increase of bacterial growth in comparison with BV-2 which seems to restrict more the intra-cellular proliferation, even if pro-infectious compounds are found in both, few overlaps are found.

Materials and methods

[1] Antibiotic activity assay (M. marinum)
$10^5$ LuxABCDE-expressing M. marinum (Arafah, Kicka et al. 2013) were transferred into each well of 96-well white plates. Bacterial growth at 32°C was monitored by measuring the luminescence in a platereader (Synergy H1) for at least 48 hours with a time point taken every 3 hours (Arafah, Kicka et al. 2013). Rifabutin was added as a control at 10 µM concentration.

$10^4$ GFP-ABD-expressing D. discoideum cells were transferred into each well of 96-well plates (Cell Carrier, black, transparent bottom from Perkin-Elmer), and allowed to attach for 20-30 min. Cell growth at 25°C was monitored by measuring the GFP fluorescence in a fluorescent plate reader (Synergy H1, company) for at least 48 hours with a time point taken every 3 hours.

A. castellanii were cultured in PYG medium in 10 cm Petri dishes at 25°C, and passaged the day prior to infection to reach 90% confluency. M. marinum were cultivated in a shaking culture at 32°C to an OD$_{600}$ of 0.8-1 in 7H9 medium. Mycobacteria were centrifuged onto a
monolayer of *A. castellanii* cells at an MOI of 10 to promote efficient and synchronous uptake. Centrifugation was performed at RT at 500 g for two periods of 10 min. After an additional 20-30 min incubation, extracellular bacteria were washed off with PYG and infected cells were resuspended in PYG containing 10 μM amikacin. $5 \times 10^4$ infected cells were transferred to each well of a 96-well plate (Cell Carrier, black, transparent bottom from Perkin-Elmer) with preplated compounds and DMSO/rifabutin controls. The course of infection at 25°C was monitored by measuring fluorescence in a plate reader (Synergy H1, BioTek) for 72 hours with time points taken every 3 hours. Time courses were plotted and data from all time points were used to determine the effect of compounds versus vehicle controls. To take into account possible autofluorescence of the compounds, RFU data of the first time point were subtracted from all time points. Cumulative curves were calculated. The activities of the compounds were determined by analysing maximum difference of compound cumulative curve to the 12-16 vehicle controls.

**[4] BV2 cells infection assay**

BV2 cells were cultured in DMEM medium supplemented with FCS in 10 cm Petri dishes at 37°C. The day prior to infection, cells were trypsinized, passaged and transferred to 96-well plates (black transparent bottom from BD Falcon), so as to reach 60-70% confluency the day of the experiment.

*M. marinum* were cultivated in a shaking culture at 32°C up to an OD600 of 0.8–1 in 7H9 medium supplemented with OADC. Mycobacteria were centrifuged onto preplated BV2 cells at an MOI of 3 to promote efficient and synchronous uptake. Centrifugation was performed at RT at 500 g for 10 min in a clinical centrifuge. After an additional 20 min incubation, extracellular bacteria were washed off with DMEM and infected cells were resuspended in DMEM supplemented with FCS. Compounds from the GSK TB set were added to a 30 μM final concentration. Infected cells were incubated at 32°C. The course of infection was monitored by high-content microscopy with time points taken every 7-14 hours.

**High Content microscopy**

Infected cells were monitored in 96-well plates (black, transparent bottom from BD Falcon). Recording of transmitted light and GFP fluorescence data were performed using ImageXpress Micro XL Widefield High content Screening System (20x 0.75 NA, air).

Quantification of the image fluorescence intensity was used to determine the effect of compounds versus vehicle controls. The activities of the compounds were determined by
analysis of the maximum difference of fluorescence intensity at day 3 post-infection compared to 6 - 16 vehicle controls.

High Content microscopy were used for conformation of IC50 data generated by single point measuring fluorescence in a plate reader (Synergy H1, BioTek).

[5] IC50 determination

Compounds of interest were assayed at a range from 0.01 to 20 µM concentration in A. castellanii – M. marinum model, last data points values at 3 days post-infection were used to built dose response curve in Graph Pad Prism to determine IC50. A mean value from at least two biological replicate is given for each compounds.

References :


A
Antibiotic assay
plate 1

B
Growth assay
plate 1
Figure 1. A. Growth assay of *M. marinum* expressing GFP in the presence of 10 GSK compounds. Normalized fluorescence intensity is measured. B. Growth assay of *D. discoideum* expressing GFP-ABD. Normalized fluorescence intensity is measured. C. *A. castellanii* and *M. marinum* infection assay. Normalized fluorescence intensity is measured.
Figure 2. High-content microscopy of GSK2 compounds. BV2 cells are infected with *M. marinum* expressing *GFP*. Measurement is taken 50 hours post infection.
Appendix 1. Compounds of GFK chemical library that do not have bactericidal activity on *M. marinum*. 10 μM compound were added.
Appendix 2. Family of compounds that display strong antimycobacterial activity in BV2 assay.

Appendix 3. Structure activity relationship studies. 19 chemical derivatives of similar chemical scaffold were analyzed.
Appendix 4. IC50 calculation of GSK compounds.

\[ R^2 = 0.804 \]
\[ Q^2 = 0.073 \]

Appendix 5. Kernel-based partial least squares linear regression graph based on IC50 values.
Appendix 5. BV2 growth inhibition assay. Dotted areas indicate high cell numbers.

Figure 3. Proposed model of pro-infectious activity of antibiotics. A. Strong antitubercular compound kills bacteria inside the host and cures the infection. B. Not effective compound does not prevent mycobacterial growth. Bacteria are proliferating and killing the host. Bacteria are losing reservoirs of infection and access to host resources. C. Weak antibiotic decreases the fitness of Mycobacterium thereby increasing the fitness of the host. Mycobacteria can proliferate, use host resources and spread the infection without killing the host.
Table 1. Summary of the activities detected in GSK TB-set screening. Compounds were tested in antibiotic assay with the use of GFP-expressing *M. marinum*. Growth inhibition and cytotoxicity were addressed with GFP-ABD expressing *D. discoideum* growth assay. Infection assays were performed in *A. castellanii*/*M. marinum* and BV2 microglial cells/*M. marinum* host-pathogen model systems. The screening were performed for 72 hours. Mean values of fold increase are presented, data is normalized to vehicle control. Experiments were done in biological triplicates for antibiotic and growth assays and *A. castellanii*/*M. marinum* assay and in duplicates for microglial cells/*M. marinum* assays. Correspondent standard deviations are indicated. Anti-infectives are labeled in orange, pro-infectives are labeled in green. Cytotoxic compounds are labeled in red.

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Book Chapter. Setting Up and Monitoring an Infection of
_Dictyostelium discoideum_ with Mycobacteria

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VT’s contribution: Synergy Mx Monochromator-based multi-mode microplate reader (Biotek) maintenance, development and optimization of fluorescence-based _D. discoideum_ infection assay, cell cultivation.
Setting Up and Monitoring an Infection of *Dictyostelium discoideum* with Mycobacteria

Sonia Arafah*, Sébastien Kicka*, Valentin Trofimov, Monica Hagedorn, Nuria Andreu, Siouxsie Wiles, Brian Robertson, and Thierry Soldati

Abstract

*Mycobacterium marinum* is the causative agent of fish and amphibian tuberculosis in the wild. It is a genetically close cousin of *Mycobacterium tuberculosis*, and thereby the infection process remarkably shares many of the hallmarks of *M. tuberculosis* infection in human, at both the cellular and organism levels. Therefore, *M. marinum* is used as a model for the study of mycobacterial infection in various host organisms. Recently, the *Dictyostelium–M. marinum* system has been shown to be a valuable model that recapitulates the main features of the intracellular fate of *M. marinum*, including phagosome maturation arrest, as well as its particular cell-to-cell dissemination mode. We present here a “starter kit” of detailed methods that allows to establish an infection of *Dictyostelium* with *M. marinum* and to monitor quantitatively the intracellular bacterial growth.

**Key words** Phagocytosis, Phagosome maturation, Mycobacteria, Intracellular infection

1 Introduction

*Dictyostelium discoideum* is an amoeba model that has been used for decades to investigate development, morphogenesis, social behavior, chemotactic cell motility, and many other aspects of cell biology (1). Its fully sequenced and annotated haploid genome (2), associated with genetics tool, easy manipulation, and real-time live imaging, makes the social amoeba a remarkable experimental model organism.

*Dictyostelium* naturally grazes by extensive phagocytosis on a broad variety of soil bacteria including both Gram-negative and Gram-positive bacteria. The ability of *Dictyostelium* cells to grow

*Sonia Arafah and Sébastien Kicka contributed equally to the work.*
on a bacterial lawn has been used to discriminate between “commensal” and pathogenic bacterial species (3), as no plaque formation is observed on the latter (4, 5). Moreover, signalling pathways and key regulators needed for host–pathogen interaction (from adhesion and phagocytosis to intracellular cycle) have been conserved between specialized phagocytic cells as evolutionary distant as Dictyostelium and macrophages (6, 7). So far, Dictyostelium has been used as a powerful model for infection with human pathogenic bacteria such as Legionella, Klebsiella, Mycobacteria, or Pseudomonas species (8–12); Vibrio cholerae (13); Salmonella typhimurium (14); and even fungi such as Cryptococcus (15). Preliminary results have also been reported for Neochlamydia and Parachlamydia sp. and Neisseria meningitidis (16, 17).

Here we will summarize insight gained from the use of Dictyostelium as a host for the intracellular bacterial pathogen Mycobacterium marinum. M. marinum is a pathogenic bacterium that mostly infects fish and amphibians. As a close cousin of Mycobacterium tuberculosis, M. marinum shares most hallmarks of pathogenicity and induces very similar lesions (18, 19). First, it has been observed that M. marinum can efficiently replicate within Dictyostelium at a low temperature compatible with amoeba health/fitness (25°C) (20). The early stages of infection with M. marinum have been well described, using colony-forming unit (CFU) counting, fluorescence-activated cell sorting (FACS) analysis, and fluorescence microscopy observations (21). During infection of Dictyostelium, M. marinum is able to arrest or bypass phagosome maturation, in a way very similar to the course of infection in macrophages. M. marinum, like M. tuberculosis, manipulates the phagosome composition, essentially by preventing its acidification by the V-ATPase, recruitment and activity of which are required for proteolytic enzyme activity (22). Recruitment of the vacuolar H+ATPase is strongly decreased compared to the compartment harboring nonpathogenic mycobacteria, and becomes practically undetectable at 6 h post infection (hpi) (21). Moreover, it has been shown that M. tuberculosis also uses detoxifying enzymes as superoxide dismutase (SOD) and catalase to fight against reactive oxygen species (ROS) released by the host macrophage inside the phagosome (23). Recently, the ionic composition of the phagosomal lumen in presence of mycobacteria has been investigated more thoroughly and revealed some surprises. Indeed, remarkably, while some metal ions such as iron are depleted from the phagosome, likely because they serve as essential coenzymes in bacterial metabolism, others such as the toxic heavy metals copper and zinc are released inside the phagosome at intoxicating levels (reviewed in ref. 24).

In addition, niche rupture has been observed (21), allowing M. marinum to escape to the cytosol, as seen in macrophages (25). Finally, live observation confirmed that M. marinum but also
Infection of *Dictyostelium* with Mycobacteria

*M. tuberculosis* can egress from the host cell via an actin-based mechanism. This exit process called “ejection” is distinct from exocytosis and does not cause cell lysis (26).

*Dictyostelium* is thus a valuable model for *M. marinum* infection studies. Here, we will present methods to monitor and quantitate intracellular growth of *M. marinum* within *Dictyostelium*, using fluorescent or luminescent bacteria and host markers. For a better understanding of the use of the flow cytometry method, more details and explanations can be found in Chapter 21 of this book.

## 2 Materials

### 2.1 Buffers and Chemicals

All solutions are prepared in double distilled water unless otherwise indicated.

1. **HL5c medium including glucose** (Formedium): resuspend 26.55 g of powder in 1 L of deionized water. Autoclave or filter-sterilize (see Note 1).

2. **BBL Middlebrook OADC** (supplement for mycobacterial cell culture, Becton Dickinson). 500 mL contain 0.6 mL of oleic acid, 50 g of bovine serum albumin, 20 g of dextrose, 0.03 g of catalase, 8.5 g of NaCl. Filter-sterilize and store at 4°C.

3. **Middlebrook 7H9 broth** (Difco): dissolve 4.7 g of powder in 900 mL of deionized water. Add 500 μL Tween 80 (0.05%) and 2 mL of glycerol (0.2%) and autoclave. Let the medium cool down and add 10% v/v of BBL Middlebrook OADC. Store at 4°C.

4. **Mycobacterium 7H11 agar** (Difco): dissolve 21 g of powder in 900 mL of deionized water containing 5 mL of glycerol and autoclave. Let the medium cool down and add 10% v/v OADC.

5. **PBS**: 50 mM KH₂PO₄, 150 mM NaCl, pH 7.2.

6. **PBS-Triton**: PBS containing 0.1% Triton X-100.

7. **PBS-Tween**: PBS containing 0.2% (v/v) Tween 80.

8. **Soerensen buffer (SB)**: 15 mM KH₂PO₄, 2 mM Na₂HPO₄, pH 6.0.

9. **Soerensen/sorbitol buffer (SSB)**: Soerensen buffer containing 120 mM sorbitol.

10. **SSB + azide**: SSB containing 5 mM sodium azide.

11. **Penicillin/streptomycin (PS) 100× stock solution**, 10,000 U and 10,000 μg/mL, respectively (Gibco).

12. **Kanamycin**: prepare a 1,000× stock solution by dissolving 30 mg of powder in 1 mL of deionized water. Filter-sterilize and store at −20°C.
13. Hygromycin: prepare a 1,000× stock solution by dissolving 100 mg of powder in 1 mL of deionized water. Filter-sterilize and store at −20°C.

14. Neomycin (G418): prepare a stock solution by dissolving 10 mg of powder in 1 mL of deionized water. Filter-sterilize and store at −20°C.

15. Amikacin: prepare a 2,000× solution by dissolving 11.7 mg of powder in 1 mL of deionized water. Filter-sterilize and store at −4°C.

2.2 Equipment

1. Glass beads, 5 mm diameter (Sigma).

2. YG-beads: Fluoresbrite® YG Carboxylate Microspheres 4.50 μm diameter, 4.99×10⁸ particles/mL (Polysciences). Store at 4°C. They should be diluted 1:10 in Soerensen and sonicated 5 min just before use. YG-beads are excited by the 488 nm argon laser of the flow cytometer and detected in the FL1 channel.

3. 10-mL syringes (BD).

4. Anel blunt needle 25 G×3/4″, 0.5×18 (Neoject).

5. FilterTop 500-mL, 0.22-μm polyethersulfone (PES) (TPP).

6. 10-cm cell culture dishes, tissue-culture treated polystyrene (BD Falcon).

7. Petri dishes (Sterilin®).

8. 6-well plates (BD Falcon).


10. CellCarrier-96 Black, optically clear bottom, tissue-culture treated, sterile, 96-well plates with lid (Perkin Elmer).

11. Gas permeable moisture barrier seal (Bioconcept).

12. µ-dish, 35-mm high (Ibidi).

13. FACS tubes (BD Biosciences).

14. Tabletop centrifuge.

15. Beckman Coulter Allegra 6R centrifuge.

16. Incubator at 32°C.

17. Shaker at 32 and 25°C.

18. Bath sonicator.

19. Cell culture inverted microscope with a 40× phase contrast objective.

2.3 General Bacterial and Cell Culture

1. D. discoideum: the laboratory wild-type Ax2 strain is grown axenically in HL5c containing 1× PS in 10-cm dishes at 22°C. The GFP-ABD (pDXA-GFP-ABD120 (27)) expressing Ax2 strain is grown in presence of 1× PS and 5 μg/mL G418.
2. *M. marinum*: this bacterium is a Biosafety Level 2 organism and requires special practices and biosafety equipment when manipulated (see Note 2). Three strains are used in the following assays and were obtained by transformation of the *M. marinum* M strain with the pMV306-lux plasmid (28) rendering cells luminescent, with GFP expression vector (from Dr. L. Ramakrishnan) or with the pCherry10 plasmid (29) to obtain green or red fluorescent bacteria. The mycobacteria strains described are available from the authors.

(a) Make a first shaking culture at 150 rpm and 32°C, from a −80°C glycerol stock in tubes containing 5 mL of 7H9 supplemented with kanamycin (25 μg/mL) or hygromycin (100 μg/mL) (respectively, for luminescent and fluorescent bacteria).

(b) Large cultures can be prepared by growing bacteria in flasks in the presence of glass beads to minimize clumping, at 32°C in shaking culture (150 rpm) in 7H9 broth containing kanamycin or hygromycin, as appropriate. Mycobacteria are grown to an OD$_{600}$ of 1 (5 × 10$^8$ bacteria/mL).

### 2.4 Preparation of the Cells for Infection

The day before infection, seed *Dictyostelium* cells on 10-cm dishes containing HL5c without PS (see Note 3) in order to obtain 80% confluent plates the day of infection.

### 2.5 Instruments to Monitor Infection

1. Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (Biotek) is a temperature-controlled machine used to measure fluorescence (from 250 to 900 nm) and luminescence signals.

2. Luminoview LV200 (Olympus) is a bioluminescence imaging microscope (see Note 4) that is controlled by the SlideBook 5.0 software and that can be used for long-term live imaging of cells. A 63× objective is used to monitor infection of *Dictyostelium* with luminescent bacteria.

3. Flow cytometer (see Chapter 21, Subheading 2.5, step 1, for details).

### 3 Methods

#### 3.1 Infection Assay

1. Wash mycobacteria twice in HL5c medium and disrupt residual clumps through passages through a 25-gauge blunt needle.

2. Add bacteria homogeneously onto 10 cm dishes of 80% confluent adherent *Dictyostelium* cells (around 5 × 10$^7$) at multiplicity of infection (MOI) of 10.

3. Centrifuge dishes at 500 × g twice for 10 min using the Allegra 6R centrifuge. Turn the dish 180° between the two
centrifugation steps to avoid massive accumulation of cells and bacteria on one side of the dish.

4. Incubate cells at 25°C for an additional 10–20 min. Then, excess extracellular bacteria are carefully removed by washing with HL5c as many times as necessary by carefully adding/pipetting fresh HL5c media without detaching Dictyostelium cells (see Note 5).

5. An antibiotic needs to be added at a concentration that completely inhibits extracellular proliferation of bacteria, but is nevertheless permissive for intracellular bacteria growth. For example, PS (5 μg/mL streptomycin, 5 U/mL penicillin) or amikacin (10 μM) (20, 30) can be used.

6. Detach cells from the dish, count, and resuspend them in HL5c to a density of 1 x 10^6 cells/mL and finally dispense in a 6-well plate (5 mL per well) and leave in a 25°C shaking incubator for the required period of time (see Subheadings 3.2.1 and 3.2.3).

### 3.2 Monitoring Infection

Several techniques have been successfully developed to monitor bacterial survival in infected cells and used during experimental infection of Dictyostelium with M. marinum. Quantification of CFU on agar plates is a standard way to follow bacterial growth and/or survival over time. New reporter technologies (fluorescence and luminescence) have been recently developed in M. tuberculosis and successfully applied to M. marinum.

#### 3.2.1 Quantification of CFUs

The following steps are applied at all time points [0.5, 12, 21, 37, and 43 h post infection (hpi)] during the course of one cycle of infection setup as described under Subheading 3.1. Fresh 7H11 agar plates should be used. Thus, on the day of infection, pour Petri dishes with 7H11 agar prepared as described under Subheading 2.1, item 4, and allow them to dry.

1. Mix the suspension of infected cells contained in the 6-well plate by carefully pipetting up and down and transfer 300 μL of the mixture to an Eppendorf tube containing 500 μL of HL5c without PS.
2. Centrifuge the cells at 10,600 x g for 4 min at room temperature in a table top centrifuge.
3. Resuspend the cell pellet in 300 μL of PBS-Triton X-100 and incubate for 10 min at room temperature.
4. Vigorously pipette the lysed cells up and down.
5. Dilute the lysate by preparing four serial dilutions: mix 30 μL of the lysate with 270 μL of PBS-Tween, homogenize, and mix 30 μL of this diluted lysate with 270 μL of PBS-Tween. Repeat two more times (see Note 6).
For each suspension of infected cells, divide a plate of 7H11 agar in quarters and spot 3 aliquots of 15 μL for each dilution per quarter plate. Do not spread the drops but let them dry instead.

Incubate at 32°C in a humidified box until colonies appear (see Note 7).

Count the colonies for each drop and calculate the mean for each dilution. Plot the results on a graph showing the number of CFUs over time (see Fig. 1).

A microplate reader can be used to monitor growth of fluorescent *M. marinum* within *Dictyostelium* during the course of infection. We present here methods to monitor bacteria and host growth from an infection of *Dictyostelium* cells expressing a GFP marker with an *M. marinum* strain expressing mCherry by quantitating their fluorescence as a function of time.

To estimate the average number of mycobacteria per cell, a serial dilution of fluorescent bacteria of known concentration (given by OD₆₀₀ measurement, see Subheading 2.3, item 2) in HL5c is placed in a 96-well plate and their fluorescence value obtained from the microplate reader using λ(excitation/emission) 587/610 nm for mCherry (see Notes 8 and 9). Knowing the number of infected...
cells added to each well, and their associated mCherry fluorescence that is directly proportional to the number of bacteria, it is possible to calculate the average number of bacteria per cell. Alternatively, or complementarily, the proportion of infected cells and the number of bacteria/cell can be directly obtained by using a fluorescence microscope.

**Control Wells**

At least one well of each of the following controls should be included with every experiment:

1. One well is dedicated to medium only and used to obtain the background fluorescence value.
2. One well with noninfected cells to check cells’ auto-fluorescence at each excitation/emission wavelength.
3. One well filled with mycobacteria (~2 × 10^5) in HL5c without antibiotic to monitor bacterial growth.
4. One well of mycobacteria (~2 × 10^5) in presence of antibiotic used to prevent extracellular growth to verify antibiotic efficiency.

**Plate Preparation and Reading and Data Analysis**

1. Fill a 96-well plate suitable for fluorescence plate reading with 100–200 μL of a suspension of infected cells; each well should contain 1–2 × 10^4 *Dictyostelium* cells to record up to 48–72 hpi before cells become confluent.
2. Seal plates with a gas permeable film that avoids evaporation and well cross-contamination by overspill but allows oxygen supply.
3. Set up the temperature of the plate reader at 25°C (permissive for *Dictyostelium* and *M. marinum* growth).
4. Use bottom-reading setup of the microplate reader (this reading mode is best adapted to cell-based assay even though top reading leads to similar results)
5. Record fluorescence values during the first 2–3 days of infection; the measurement normally starts around 30–45 min post infection (considering the time zero when bacteria are added onto the cells) (see Fig. 2).

**3.2.3 Luminescence Quantiﬁcation of the Luminescence Signal with the Synergy Mx Reader**

To measure luminescence signals, a white 96-well plate (see Note 10) and top reading are necessary. The temperature is set at 25°C. Two parameters need to be optimized according to the machine used, sensitivity, and acquisition time. If using high sensitivity, the intensities may be above a threshold of luminescence value (overflow) that will result in the inability of the reader to give a measurement of the luminescence signal. Short acquisition times may lead to inefficient detection of the luminescence signal. The following steps are applied at all time points (0.5, 12, 21, 37, and 43 hpi) during the course of one cycle of infection.
Fig. 2 Monitoring of the *M. marinum* population by fluorescence recording and microscopy during a cycle of infection in *Dictyostelium*. (a) Fluorescence background of the HL5c medium at excitation/emission wavelengths used for mCherry (587/610 nm) and GFP (489/509 nm). (b) Growth kinetics of AX2 GFP-ABD cells infected or not infected (27), as measured by reading the GFP fluorescence intensity. (c) Growth kinetics of *M. marinum* mCherry in presence or absence of amikacin, as measured by the mCherry fluorescence intensity. (d) Intracellular growth kinetic of *M. marinum* as measured by the GFP fluorescence intensity. (e) Confocal microscopy image of AX2 GFP-ABD cells infected with *M. marinum* mCherry at 1 hpi. Arrows point to intracellular mycobacteria. The scale bar is 10 µm.
1. Mix the suspension of infected cells contained in the 6-well plates (Subheading 3.1, step 6) by pipetting up and down, and transfer 150 μL of the mixture in a well of a white 96-well plate. Also fill a well with HL5c to measure the background signal.

2. Measure the luminescence signal and subtract the background value. Plot the results in a graph showing the relative luminescence unit (RLU) over time (see Fig. 3a).
To image the cells and the luminescence signal, some important parameters need to be taken into account. The luminescence microscope should be in a completely dark room. The exposure time for low intensity luminescence usually lasts several minutes. An intensification of the signal is therefore required. However, increasing the intensification too much is associated with an increase in background noise, leading to less sharp luminescence spots. For bright-field images, short exposure times (in the millisecond range) are usually used. When using *Dictyostelium* strains expressing a fluorescent marker, no intensification is needed. Once again, exposure time in the range of milliseconds should be used.

1. Mix the suspension of infected cells contained in the 6-well plates by pipetting up and down and deposit 300 μL of the mixture in an Ibidi dish (see Note 10). Let the cells attach to the plastic dish.

2. At the microscope, focus on the cells. Then, image first the luminescence signal and then the bright-field and/or fluorescence signals if needed (see Note 11). Using ImageJ software (http://rsbweb.nih.gov/ij/), merge images (see Fig. 3b).

Infection is performed with GFP-expressing bacteria that are excited by the 488 nm argon laser of the flow cytometer and detected in the FL1 (green) channel.

1. Transfer 500 μL of the suspension of infected cells into 500 μL of SB+ azide in an Eppendorf tube.

2. Centrifuge at 15,900 × g for 4 min to pellet all the cells at room temperature.

3. Resuspend the cell pellet in 500 μL of SSB.

4. Transfer into FACS-tube and keep on ice until use.

5. Just before analysis, add 1 μL of sonicated YG-beads (see Note 12).

6. At the FACS, acquire data in two ways; count 300 beads and also 20,000 cells (see Note 13).

7. Perform data analysis with the Flowjo software (Treestar) (for details, see Chapter 21, Subheading 3.3) (see Fig. 4).

### Notes

1. It has been observed that filter-sterilized medium is less fluorescent than the autoclaved one.

2. *M. marinum* is classified as a Biosafety Level 2 (BSL2) organism. Although it does not cause a systemic disease in humans,
Fig. 4 Monitoring of the population dynamics of intracellular and extracellular *M. marinum* by flow cytometry during a cycle of infection in *Dictyostelium*. Flow cytometry analysis during an infection shows three different populations, after plotting of the side scattering (SSC) as a function of fluorescence (FL1): noninfected cells (1), cells harboring fluorescent mycobacteria (2), and fluorescent extracellular mycobacteria (3). The percentage of each population is shown inside the corresponding gate. (a) YG-beads were added to the suspension of infected cells and 300 beads were counted. This allows direct observation and comparison of the dynamics of the three populations over time. The graphs show the distribution of cells at 0.5, 12, 21, 37, and 43 hpi. No marked differences were observed between 0 and 12 hpi. Afterwards, the proportion of infected cells decreases as cells divide but the number of infected cells and total fluorescence of this population is maintained and even increased. From 37 hpi, the total fluorescence of infected cells starts to decrease and a concomitant increase of extracellular mycobacteria is observed. (b) The aliquots of the suspension of infected cells used in (a) were also subjected to counting of 20,000 cells. The proportions and the dynamic of each cell population were very similar to those obtained in (a).
because of its temperature restriction of about 32°C, it can be responsible for skin infections and granuloma at extremities. In case of infection, curing requires 6 months of treatment with standard antituberculous antibiotics. Thus, manipulation of *M. marinum* must be performed in a contained dedicated laboratory and requires specific training of personnel and appropriate safety equipment (gloves, puncture-proof gloves, blunt needles, lab coats, class 2 flow cabinets). Hazardous waste must be autoclaved in the vicinity of the BSL2 laboratory.

3. Penicillin/streptomycin and any other antibiotic (G418, hygromycin) should be omitted when preparing the cells for infection to prevent bactericidal effect on intracellular bacteria.

4. Dual-color luminescence and transmission fluorescence can also be performed, thanks to specific excitation and emission filter wheels.

5. Presence of extracellular mycobacteria can also be monitored by optical microscopy observation.

6. Tween 80 will favor a better separation of bacteria and result in a more accurate number of CFUs.

7. Development of colonies takes at least 5–7 days; therefore, humidification of the box (e.g., with wet paper towels) is highly recommended. Do not take into account drops that contain more than 100 colonies as they might not be well separated. This would lead to underestimation of the number of colonies.

8. A quick centrifugation at $600 \times g$ can be performed to rapidly bring bacteria and cells at the bottom of the well instead of waiting for them to sediment which takes around 30 min.

9. Fluorescence values of the infected cells are calculated as $\text{RFU}_{\text{read}} - \text{RFU}_{\text{background}}$. Despite variations of mCherry fluorescence value at the beginning, the growth curve should not be normalized at the starting point, because it gives too much weight to this starting condition.

10. White plates are used to measure luminescence signals as they provide maximum reflection of light but minimum autoluminescence. At the beginning of the infection, up to 300 µL of the suspension of infected cells can be used for measurement. As cells grow during the infection, half that volume should be used from 37 hpi to avoid overconfluence.

11. Luminescence images should always be taken first, as photons coming from the samples are directly detected without any excitation. If recording fluorescence images first, the excitation light might disturb the subsequent capture of luminescence signal.
12. YG-beads are used as internal standard to compare samples over time.

13. As beads are used as internal standard, counting 300 of them at each time point allows a direct comparison of the cell populations from each sample, and cell growth can be followed over time. However, counting 300 beads corresponds to a low number of cells from the suspension. For data analysis, such as determination of the proportion of infected cells or the mean fluorescence, a higher number of cells is required. Therefore, 20,000 events are counted in parallel.

Acknowledgement

The laboratory of Thierry Soldati is supported by multiple grants from the Swiss National Science Foundation.

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Infection of *Dictyostelium* with Mycobacteria

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Establishment and Validation of Whole-Cell Based Fluorescence Assays to Identify Anti-Mycobacterial Compounds Using the Acanthamoeba castellanii – Mycobacterium marinum Host-Pathogen System

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Establishment and Validation of Whole-Cell Based Fluorescence Assays to Identify Anti-Mycobacterial Compounds Using the Acanthamoeba castellanii - Mycobacterium marinum Host-Pathogen System

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Abstract

Tuberculosis is considered to be one of the world’s deadliest disease with 2 million deaths each year. The need for new antitubercular drugs is further exacerbated by the emergence of drug-resistance strains. Despite multiple recent efforts, the majority of the hits discovered by traditional target-based screening showed low efficiency in vivo. Therefore, there is heightened demand for whole-cell based approaches directly using host-pathogen systems. The phenotypic host-pathogen assay described here is based on the monitoring of GFP-expressing Mycobacterium marinum during infection of the amoeba Acanthamoeba castellanii. The assay showed straightforward medium-throughput scalability, robustness and ease of manipulation, demonstrating its qualities as an efficient compound screening system. Validation with a series of known antitubercular compounds highlighted the advantages of the assay in comparison to previously published macrophage-Mycobacterium tuberculosis-based screening systems. Combination with secondary growth assays based on either GFP-expressing D. discoideum or M. marinum allowed us to further fine-tune compound characterization by distinguishing and quantifying growth inhibition, cytotoxic properties and antibiotic activities of the compounds. The simple and relatively low cost system described here is most suitable to detect anti-infective compounds, whether they present antibiotic activities or not, in which case they might exert anti-virulence or host defense boosting activities, both of which are largely overlooked by classical screening approaches.

Introduction

Tuberculosis, a Serious Health Threat

The negative impact tuberculosis (TB) has on human health is hard to overestimate. Over one third of the world population is infected by bacteria of the Mycobacterium tuberculosis (Mtb) complex. Each year two million TB-related deaths are registered with 8 million newly infected people [1]. Despite the efforts of modern therapeutics, in nine of ten cases, Mtb manages to persist throughout the lifetime causing the risk of reinfection and reemergence of the disease [2].

The hallmark of TB is the formation of granuloma, well-organized multicellular structures primarily composed of mature macrophages and T-lymphocytes. Macrophages often develop into multinucleated giant cells and epithelioid cells. Granulomas also contain dendritic cells, neutrophils, NK-cells, fibroblasts and B-lymphocytes and are surrounded by a fibrous cuff. In addition, epithelial cells surrounding granulomas are proposed to participate in its formation. It is generally assumed that the granuloma is a host-defensive structure that sequesters and eradicates pathogenic bacteria. Although there is evidence of healed and often sterile granuloma among certain TB patients, recent findings indicated that Mtb employs a distinct mechanism of proliferation via granulomas [22]. Mtb mostly replicates in alveolar macrophages but can also be found in dendritic cells, adipocytes and type II alveolar pneumocytes [3–6].

Pathogenic mycobacteria, such as Mtb and other mycobacteria of the tuberculosis complex, but also Mycobacterium leprae, Mycobacterium marinum and Mycobacterium avium are able to manipulate a variety of processes including membrane trafficking [3,7], autophagy [8,9], signaling [10] and apoptosis [11,12]. These manipulations of its host allow the bacteria to hijack the phagosome and prevent major steps of its maturation by performing rapid exclusion of the vacuolar H-ATPases [13],

In order to find a way to counteract TB infection, considerable research efforts focus on a mechanistic study of mycobacterial virulence factors. One of them is encoded by the RD1 locus, which was first discovered by investigating the genome deletions in the attenuated Mycobacterium bovis BCG vaccine strain. Studies performed also with M. marinum found it to be the main virulence determinant [18,19]. The locus encodes a type 7 secretion system, called ESX-1 system [18]. It was shown that RD1 mutants are less effective in arresting phagosome maturation and are attenuated in infection dissemination [18,20–23].

TB Treatment, Search for New Drugs

The standard treatment for tuberculosis uses a combination of antitubercular compounds for six months or longer. The necessity of extensive treatment was elaborated after a long period of trial and error. It is now clear that noncompliance with the treatment, short-term and relaxed therapy regimens result in the emergence of drug-resistant strains. The situation has escalated even further due to emergence of multi-drug resistant (MDR) strains and, finally, extensively drug resistant (XDR) strains, and more recently some totally drug-resistant strains have been described [24]. As a consequence, the WHO reviewed the strategy to fight TB infections, leading to the establishment of “directly observed treatment short course” (DOTS) [WHO report 2011].

The newest drug for TB treatment is 30 years old, and the previously very effective streptomycin lost its efficiency against M. tuberculosis and is no longer used for therapy. Therefore, the need for new drugs has become obvious. Several reasons underlie the lack of new drugs, such as the difficulty to identify compounds that penetrate mycobacteria, because of the low permeability of the mycolate-rich cell wall or because of the low metabolic and growth rates reflected by their 24–36 hours doubling time. In addition, conventional screening approaches usually favor the search for bactericidal compounds while at the same time neglecting host-pathogen interactions.

Despite the challenges mentioned above, several drug candidates are currently under development and have a good chance to enter the market. Promising approaches for drug development include targeting synthesis of lipids as nutrients [25,26] and synthesis of mycolic acids as major components of the cell wall [27]. In the last decade, researchers have identified compounds that kill dormant bacteria by intracellular NO release, such as the bicyclic nitroimidazoles, PA-824 [28], and OPC-67683, as well as compounds that affect ATP-synthesis such as TMC207 [29] and nitrofuranylamide compounds with so far unknown mode of action [30]. Some screens revealed prodrugs that are activated by the metabolism of the host cell, such as nitroimidazopyran [31]. Potentially interesting compounds also include heterocyclic aldehydes [32], oxazole- and oxazoline-containing compounds that target iron uptake [33], and rhodanine derivatives that target the dihydroxyacetone acyltransferase [34].

Whole-cell Based Screening, a Promising Alternative to Target-based Approaches

Standard target-based approaches identified compounds that showed very high attrition rates and low numbers of validated hits against the intact live bacterium and in infection systems [35,36]. Meanwhile, a broad spectrum of new tools has become available [37]. A new trend has emerged: phenotypic screens in a whole-cell infection system [38,39]. Whole-cell screens are a promising method to provide lead-structures and identify new targets. Unlike target-based approaches they fulfill in vivo criteria such as membrane permeability and a higher activity against mycobacteria than host cells. However, whole-cell based assays typically do not easily reveal the mechanism of action. Additional mechanistic studies and rounds of structure-activity relationship investigations are required.

Establishing alternative methods to target-based screening may improve the chances to discover new sets of drugs that could be competitive with current drugs, shorten the duration of treatment, avoid significant drug-drug interactions, and successfully deal with MDR and XDR Mtb strains. The ability of whole-cell screens to detect host response in situ makes it possible to reveal not only antibiotic activities, but also anti-infective drugs. Such compounds target infection-specific biological processes, and therefore, significantly reduce the risk of acquiring resistance. A proof of feasibility for the identification of such active compounds was established in a few recent studies (reviewed in [40]). For M. tuberculosis, the list contains inhibitors of iron metabolism [41] and compounds targeting resistance to oxidative stress [34]. Moreover, whole-cell-based approaches allow detection of compounds that increase the activity of natural, host-specific innate immune defense mechanisms. This opens the possibility of discovering compounds capable of helping the host cell deal with a broad range of pathogens. For example, the cellular pool of kinases and phosphatases was shown to be the targets of defense-boosting compounds [39,42–44].

Mycobacterium marinum as a Pathogen Model for Drug Screening Purposes

As mentioned above, despite the fact that many screens resulted in the discovery of promising antimycobacterial compounds, overall screening for anti-Mtb drugs remains ineffective [29,30], raising the demand for new strategies, including the use of new and more cost-efficient host-pathogen models.

M. marinum, the closest relative of Mtb in the tuberculosis complex, is an attractive alternative model. M. marinum is a fish and frog pathogen which establishes an infection similar to human tuberculosis [34]. Bacterial growth temperature is optimal at 30°C, rendering it less dangerous for humans, as it is only capable to establish superficial skin lesions [45]. Moreover its doubling time of eight hours is much shorter than that of Mtb and M. bovis BCG, which in turn improves the speed of detection of antimycobacterial effects. For M. marinum the mechanisms of phagosome maturation arrest, as well as the activity of many virulence genes are very similar to M. tuberculosis [20,46]. M. marinum readily escapes its vacuole [47], but the efficiency and relevance of this process for Mtb is still debated [48]. The high degree of functional conservation in virulence genes supports the theory that ancient mycobacterial precursors developed the mechanisms of pathogenesis against phagocytic protozoa and that mycobacteria are now using them to hijack animal immune phagocytes [49]. Indeed it has been shown that free-living amoebae can be an environmental reservoir for pathogenic bacteria such as M. avium, M. marinum and even Mtb [50].

For M. marinum, well-developed genetic and cellular biology tools are available. These features render M. marinum extremely useful for the investigation of the mode of action of antitubercular compounds and for the validation with the M. tuberculosis model [51].

Amoebae Host Systems for Drug Screening

Among whole-cell based assays the usage of unicellular hosts is advantageous because of the ease of cultivation and manipulation important in high-throughput screening. Although protozoa do not engage in complex multicellular interactions, the high degree
of conservation of innate immune defense mechanisms renders them attractive alternative systems for experimental infection studies. Within the host one can target multiple pathways involved at different stages of the infection, including endosomal trafficking during phagocytosis of the bacteria, the autophagy pathway in the form of xenophagy, ion-pumps recruitment involved in bacteria degradation during phagosome maturation, kinases and phosphatases signaling that affect the course of infection (reviewed in [49,52], [53]).

Since the primary target of Mtb is macrophages, amoebae that are also professional phagocytes, are a rational choice to study host-pathogen interactions. Amoebae offer a well-balanced compromise between the natural complexity of the system on one side and ease of manipulation and cultivation on the other. Amoebae and macrophages possess a high degree of functional conservation in defense mechanisms against infection [54]. Many species of amoebae serve as a natural reservoir and a training field for pathogens. *Acanthamoeba* are a particularly promising genus of amoebae for screening purposes. Its environmental niches include soil, air and fresh water. Unlike *Dictyostelium discoideum*, a soil-inhabiting social amoeba that is another popular protozoan model, *Acanthamoeba* does not undergo a multicellular developmental phase, which probably renders them less sensitive to the stress factors inevitable in screening processes. *Acanthamoeba* is considered to be a natural carrier for many mycobacteria species [55], for example it was shown that 25 mycobacteria species, including non-tuberculous mycobacteria, can infect both trophozoites and cysts of *Acanthamoeba polyphaga* [56–58]. Moreover, intracellular *M. avium* within *Acanthamoeba castellanii* showed increased resistance to bactericidal compounds such as rifabutin, compared to growth within macrophages [56,59]. The ability to protect from antituberculous drugs may serve as an additional in vivo filter to subtract false-positive hits of drug screening. On the other hand, the *D. discoideum* model system has its own unique advantages, particularly a complete set of genetic tools that are extremely useful for the determination of mechanisms of action. Together with a fully sequenced and annotated haploid genome, *D. discoideum* is amenable to forward and reverse genetics. Its simplicity of cultivation makes it easily biochemically tractable. *D. discoideum* also allows excellent real-time live imaging. Taken together, both amoeba genera are useful models, each having its advantages depending on the purpose of the experiments.

In the present study we have established the *A. castellanii* – *M. marinum* host-pathogen system as a robust compound screening and validation system. Together with secondary assays using *D. discoideum* and *M. marinum*, it shows excellent promise to identify novel antitubercular hits.

Results

A Fluorescence- and Cell-based Assay to Measure Intracellular Mycobacterial Growth in *A. castellanii*

In this study, we present a fast and easy approach to identify compounds with anti-infective properties in a cellular host context. *M. marinum* is able to replicate efficiently in the free-living fresh water amoeba *A. castellanii* [60]. We therefore used *A. castellanii* to monitor intracellular growth of *M. marinum* using a fluorescence-based assay. *A. castellanii* was chosen instead of our *D. discoideum* model due to its ‘macrophage-like’ size that allows a higher level of bacteria uptake, easily detectable with our fluorescence plate reader. The protocol established uses an optimized multiplicity of infection (MOI) and is based on synchronous and homogeneous bacterial phagocytosis. Spinoculation of mycobacteria on top of a cell monolayer close to confluency maximizes host-bacteria contact and subsequent uptake and thus guarantees high reproducibility of the infection course. The percentage of infected cells at time zero and the average number of bacteria per cell is a function of the MOI (Figure 1A). Using an MOI of 10:1 ensures that a majority of cells (≈ 80%) are infected (Figure 1B) with approximately 1 to 5 bacteria per cell, and thus, this condition became our infection standard. Then, excess extracellular bacteria were carefully removed by washing with PYG medium, prior to testing compounds of interest on infected cells. [61–63]Finally, infected cells were resuspended in PYG medium containing amikacin to prevent extracellular growth of bacteria [64]. Using 10 μM amikacin prevented bacteria proliferation in PYG medium for at least three days (Figure 1C).

In order to adapt the monitoring of infection to a larger scale, a fluorescence plate reader assay was optimized for the 96-well plate format. Use of mycobacteria strains expressing fluorescent reporters has recently been validated for the quantitative measurement of bacterial mass, as an alternative to c.f.u. counting, both for microscopy on live and fixed cells and organisms, as well as higher throughputs methods such as microwell-plate readers [61–63]. The fluorescent *M. marinum* msp12::GFP strain [65] gave us the most robust readout to quantitate the increase in bacterial numbers, but other fluorescent and bioluminescent reporters can also be used [66]. As shown in figure 1D, the plating density of initially infected cells (obtained with an MOI 10:1) was a parameter that greatly impacted intracellular bacterial growth. As indicated at the right of the graph, the fluorescence fold increase at 3 days post infection (DPI) was low at high cell density (1.2 and 1.6 for 2*10^5 and 1*10^5 cells/well, respectively) and bacterial growth kinetics reached a plateau after 30–40 hours post infection (HPI). In contrast, lower densities that allowed host cells to grow for at least two days before reaching confluency, resulted in a higher bacterial expansion (4–6 fold increase with 2 to 5*10^4 cells/well). Therefore, we decided to plate between 1 and 5*10^4 infected cells in each well of the 96-well plate.

We validated our assay by monitoring *A. castellanii* infection with the non-pathogenic mycobacterium, *Mycobacterium smegmatis*, and an avirulent mutant, *M. marinum*-L1D [63]. As presented in figure 1E, the total fluorescence of GFP-*M. smegmatis* decreased over time, indicating that the bacteria are killed and digested by the amoeba. In addition, similarly to its fate in zebrafish and macrophages, the *M. marinum*-L1D mutant was not able to replicate in *A. castellanii*. Similar results have been reported using the *D. discoideum* host [47]. However, *M. marinum*-L1D’s fluorescence remained stable, indicating that *A. castellanii* appears unable to fully digest this strongly attenuated *M. marinum* strain.

Under our conditions, *A. castellanii* infection with *M. marinum* appeared to severely decrease the growth and/or survival of the amoeba. Inspection of the wells by phase contrast microscopy showed that infected *A. castellanii* cells did not reach maximal confluency at 3 DPI, concomitantly with a notable accumulation of extracellular bacteria at the well centre (Figure 1F, arrow). Further inspections showed that heavily infected and dead amoebae, as well as normal giant cells are often observed during the late phase of infection (Figure 1G, arrowhead and arrow, respectively). Lethality induced by *M. marinum* infection has been reported in many animal systems such as the *Drosophila* larvae [67], leopard frog [68], and in macrophages [69]. Amoeba lysis has also been mentioned as a result of infection with other intracellular pathogens, such as *Legionella pneumophila* [70].
Figure 1. Mycobacteria infection of A. castellanii. A. Confocal (top) and brightfield (bottom) pictures of A. castellanii infected with GFP-expressing M. marinum at different MOI. Scale bar, 10 μm. B. Corresponding percentage of infected cells under the three MOI conditions, error bars represent the standard deviation from technical replicates (three microscopy fields and at least forty counted cells) of one representative experiment. C. Growth kinetics of M. marinum msp12::GFP in PYG medium supplemented with 10 μM amikacin, representative experiment from a series with
similar outcome. D. Growth kinetics of GFP-expressing *M. marinum* within *A. castellanii* plated at different densities, measured by total fluorescence intensity. The standard deviation derived from the technical mean of eight microwells. The values on the right represent the fold fluorescence increase between 2 and 68 hours post infection. E. Representative experiment of growth kinetics of GFP-expressing *M. smegmatis*, *M. marinum* WT and L1D mutant strains within *A. castellanii*, measured by fluorescence intensity. F. Brightfield microscopy of the cells at the bottom of a microwell. Infected cells at 3 DPI under control conditions and non-infected cells. Scale bar is 100 μm. G. Phase contrast (top) and confocal (bottom) pictures of *A. castellanii* infected cells with GFP-expressing *M. marinum* at three days post-infection. Infected giants cells (arrow), and some dead cells (arrowhead) are observed. Scale bar, 10 μm.

doi:10.1371/journal.pone.0087834.g001

**Drug Validation**

As a close cousin of *M. tuberculosis*, *M. marinum* is sensitive to most standard antibiotics used to treat tuberculosis, and also used to validate various screenings protocols [62,71]. In our host-pathogen infection model, most first line anti-tubercular drugs are efficient. Isoniazid (INH), ethambutol and rifabutin were active at 30 μM and efficiently blocked intracellular mycobacterial growth, only pyrazinamide was not active (Figure 2A). A rifamycin family derivative, rifabutin, was the most potent antibiotic in our infection assay with an MIC around 0.25 μM (Figure 2B). We also confirmed that rifabutin curing of an *M. marinum* infection occurred in a dose-dependent manner, and was consistent with confocal microscopy observations of infected cells (Figure 2B and C). At three days post infection, the intracellular bacteria load drastically diminished in presence of rifabutin, as reported by the total fluorescence intensity of bacteria inside infected cells (Figure 1). It is notable that rifabutin action on infection also restored host cell growth in a dose-dependent manner, as judged by the host cell density in Figure 2C. Finally, we assayed a panel of known specific anti-tubercular compounds and broad-spectrum antibiotics to treat another intracellular pathogen infection, *L. pneumophila*, in the *A. castellanii* host (Figure 3A). Despite the common ability of these pathogens to avoid phago-lysosomal fusion, the *Legionella*-containing vacuole (LCV) differs from the *M. marinum* phagosome-derived compartment, and therefore, as expected, most of the anti-tubercular antibiotics have no effect on *L. pneumophila* replication. Only drugs such as floxacins [72] and rifampin [73], already reported to be active against *L. pneumophila*, cure *A. castellanii* infected cells.

Because amoebae naturally graze on most innocuous bacteria but fail to grow on pathogenic bacteria [74], this discriminating ability can be used in an alternative screening assay to test compounds’ ability to restore amoeba growth on a mixture of pathogenic mycobacteria with non-pathogenic *Klebsiella pneumoniae* [75]. As shown in figure 3B, even though this assay is conceptually different from the GFP-based detection assay, it also detects specific anti-myobacterial antibiotics. However, broad-spectrum antibiotics, such as streptomycin or high concentrations of an anti-tubercular such as isoniazid, eradicate all bacteria and therefore, do not allow *D. discoideum* to generate phagocytic plaques.

**Assay Suitability for Drug Screening and Data Analysis**

Next, we sought to adapt the GFP-based intracellular growth assay for medium-throughput analysis using 96-well plates. Border wells are dedicated to controls needed to test bacteria fitness and amikacin efficiency (Figure 4A). As rifabutin efficiently cures the infection, we decided to include it in the experimental plate design as positive control. Wells containing DMSO (at 1%, as compound carrier) and rifabutin (10 μM) are used as negative and positive controls, respectively. Therefore, 64 compounds can be tested per 96-well plate. We next measured the quality of our screening protocol to detect potential hits by calculating a Z factor that is a usual parameter to assess screen robustness [76]. From a single standard infection, cells were dispensed in 96-wells, half containing DMSO and half containing 10 μM rifabutin, as shown in figure 4B. The assay sensitivity is high as both controls are clearly separated and, as attested by the standard deviation of the mean, variability between identical wells is low. In both cases, the end-point fluorescence values at 3 days post infection (DPI) were normally distributed. Overall assay robustness is attested by a Z factor score of 0.74 that is excellent for an ‘in vitro’ biological assay, and allows to initiate compounds screening with reasonable confidence.

To allow direct comparison from several plates and multiple infection rounds, raw fluorescence kinetic data (figure 4C, left curve) were transformed. First, to integrate the entire history of the growth kinetics, cumulative curves were built rather than simply using the endpoint measurements were built, and the first point was standardized to 0 by subtracting the value at time zero from each data point (Figure 4C, middle) were transformed as follows. Second, the last value point was standardized to 1 for the mean value of the DMSO controls (Figure 4C, right). Next, normalized cumulative values were ranked relative to the increase or decrease of bacterial growth compared to the DMSO. Representative results from a single experimental plate are presented in figure 4D. The plot represents the difference between the DMSO mean value and each compound of interest. The significance of a compound’s effect was first statistically assessed by its difference from the DMSO control, a difference of more than two or three standard deviations of the DMSO mean being considered significant. Moreover, the strength of inhibition or promotion of bacterial growth was measured by its relative normalized score compared to 1 (DMSO).

**Workflow and Secondary Assays**

In order to successfully conduct a screen to identify potential compounds of interest in a complex biological assay, secondary assays are often established and implemented prior to further structure activity relationship studies. In our specific assay setting, two further and major pieces of information can be easily extracted: compound toxicity to the host alone, and antibiotic effect on the bacterium *in vitro*. A basic representation of the screen workflow is presented in figure 5A. The effect of the hit compounds on the host growth and health was tested in the plate reader format with a *D. discoideum* strain expressing a fluorescent reporter, GFP-ABD [77]. For example, we monitored the growth kinetic of this strain in the presence of a classical anti-tubercular antibiotic, ethambutol, and a toxic compound, nicotin (Figure 5B). Ethambutol produced no detectable effect on *D. discoideum* growth, as also observed with most classical antibiotics tested so far, whereas nicotin affects cell growth in a dose-dependent manner (Figure 5B).

A similar assay was used to test the antibiotic activity of the hit compounds directly on *M. marinum* in its standard culture broth (7H9). For example, fusidic acid, a second line antibiotic used for tuberculosis treatment was tested at various concentrations on extracellular and intracellular *M. marinum* (Figure 5C). In both cases a half inhibitory concentration (IC50) can be calculated. IC50 values obtained with a small collection of classical anti-tubercular antibiotics are presented in Table 1. Overall, the data highlight a
shielding effect of the amoeba host, protecting the mycobacteria from the antibiotics, as previously demonstrated using *M. avium* [59]. In our assay, only rifabutin showed a better effect when tested as anti-infective *in vivo* rather than as antibiotic *in vitro*. This phenomenon is probably explained by its higher liposolubility, which likely enhanced membrane permeability and consequently increased its concentration inside the host cell [78].

**Discussion**

In the present study, we detail a screening method to detect anti-tubercular compounds acting in the context of infected amoeba host cells. We first developed and validated suitable conditions for medium throughput screening (MTS). We used fluorescent mycobacteria to monitor intracellular growth in real time by recording fluorescence increase in a 96-well microplate reader. So far, the use of fluorescent mycobacteria is a consensus to avoid the counting of colony forming units, which is hardly compatible with MTS procedures, and allow a fast and reliable way to measure bacterial growth. However, some obvious limitations are due to this detection method. There is no direct way to known how healthy the remaining bacteria are, whose fluorescence remains stable over time. Presently, only a clear decrease in fluorescence intensity, as observed with rifabutin, reflects of a probable bactericidal effect. The long half-life of GFP and the resistance of mycobacteria to cell lysis conferred by their

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**Figure 2. Effect of antibiotics on intracellular growth of *M. marinum* during an infection.** A. Intracellular growth kinetic of GFP-expressing *M. marinum* measured by fluorescence intensity in the presence of 30 μM of first-line antibiotics. B. Intracellular growth kinetics of GFP-expressing *M. marinum* measured by fluorescence intensity, in the presence of different concentrations of rifabutin. A and B are representative experiments from a series with similar outcome. C. Effect of rifabutin on *M. marinum* growth during an infection. Brightfield (top) and spinning disc confocal (bottom) imaging of *A. castellanii* infected with GFP-expressing *M. marinum* in the presence of the indicated concentrations of rifabutin, 72 hours post infection. doi:10.1371/journal.pone.0087834.g002
particularly thick cell wall partly explain this phenomenon. Moreover, with this readout, compounds that interfere with cell metabolism, expression of the reporter, or quench its fluorescence can be identified as false positives.

Additionally, introducing host cell-specific fluorescent markers allows the dissection of compound effects on various cell processes like phago-lysosomal trafficking, autophagy induction or lipid storage. Altogether, these fluorescent cell-based approaches can

Figure 3. Effect of antibiotics on intracellular growth of *L. pneumophila* during an infection and in a ‘phagocytic plaque assay’. A. Left, representative experiment from a series with similar outcome of intracellular growth kinetic of GFP-expressing *L. pneumophila* measured by fluorescence intensity in the presence of 30 µM of antibiotics. Right, normalized intracellular bacterial growth (DMSO = 1) in A. castellanii, error bars represent the standard deviation from biological triplicates. B. Ability of *D. discoideum* DH1 strain (1000 cells/well) to grow on a bacterial lawn composed of *M. marinum* and *Klebsiella pneumoniae* (2:1 ration) after seven days and in presence of antibiotic compounds.
doi:10.1371/journal.pone.0087834.g003
Figure 4. Screening protocol for anti-tubercular compounds. A. Scheme of the plate design in 96-well format for compounds screening. B. Left, representative experiment of intracellular growth kinetic of GFP-expressing M. marinum measured by fluorescence intensity obtained from one control 96-well plates in presence of DMSO (N = 48) or 10 μM of rifabutin (N = 48). The small graphs on the right represent the normal distribution of the fluorescence difference between 2 and 72 hours of infection for DMSO and rifabutin. Consequently, the Z factor of this experiment was 0.74. C. For the statistical analysis, the data are treated in three steps. First, from the raw data, the value at 68 hours is subtracted from all others and a cumulative fluorescence curve is drawn. Then, the curves are normalized to the DMSO standard control. Graphs are representative of one experimental screening plate. D. Differential fluorescence values obtained from one experimental plate with diverse compounds are plotted. Horizontal bars represent 3-fold standard deviation from the DMSO mean. The green dot corresponds to the normalized DMSO controls; the orange dot to a putative pro-infectious compound; the black dot to the average of the rifabutin controls; the two red dots to two putative anti-infective compounds. The graph is representative of one experimental screening plate.

doi:10.1371/journal.pone.0087834.g004
easily be adapted to monitor multiples parameters in a high-content microscopy approach [38]. Therefore, integration of these secondary readouts can provide preliminary knowledge about the mode of action of a compound [39].

As expected, the initial conditions of the infection largely influence the kinetics of bacterial growth. Multiple parameters (percentage of infected cells, cell density, host and bacteria physiological state) modulate the final apparent increase over almost a log range (data not shown). We clearly observe that exponentially growing host cells facilitate bacterial expansion, whereas high confluence partially suppresses it. These findings suggest that cell-to-cell transmission, as observed in D. discoideum, is also a significant parameter in A. castellanii infections [47].

Surprisingly, depending on the initial bacterial load, the mycobacteria infection leads to host cell death between two to five DPI. Significant cytotoxicity towards the host cell has not been reported during D. discoideum infection [79], but was previously noted in macrophages infected by M. tuberculosis and M. marinum at high MOI and under conditions where phagosomal rupture occurred [69,80]. Modulation of apoptotic and necrotic cell death has been reported in macrophages infected with virulent and avirulent strains of M. tuberculosis [81]. However, in the absence of caspase-dependent apoptotic pathways in amoebae, infection by M. marinum likely leads to the necrotic and/or autophagic cell death, mechanistically perhaps similar to the pathways documented in the model amoeba D. discoideum [82].

IC₅₀ determination of antibiotics in extracellular conditions and within the amoeba host emphasizes the shielding role of A. castellanii, as previously shown for M. avium [59]. This capacity of A. castellanii to protect against antibiotics may be due to a lower
membrane permeability and/or enhanced host efflux pump mechanisms that purge intracellular drugs. This observation implies that our assay operates at high stringency to select hit compounds with low IC50. Even if direct comparison of anti-tubercular intracellular IC50 obtained in various studies is difficult, because of the differences in conditions used (MOI, time point), the compartment in amoebae. As discussed in a study reporting about published about the pH inside the mycobacteria-containing vacuole, acidification could lead to a poor conversion of the pro-drug into its active form, and thus might explain the inefficiency of pyrazinamide in our system. In addition, a recent paper Ahmad et al [87] showed that the minimum inhibitory concentration of pyrazinamide against M. tuberculosis and M. bovis is high. Finally, several Mycobacteria strains such as M. canetti are naturally resistant to pyrazinamide [86], as well as clinical isolates of M. kansasi and M. marinum [87]. Therefore, a higher concentration of pyrazinamide could be needed to impact on M. marinum replication in our system. From our standard experimental data, we calculated a Z factor that lies between 0.6 and 0.8, values that are commonly accepted as highlighting the robustness of an assay [76]. Moreover, we also present here the flow of statistical data analysis that guides us to identify primary hits. Transformations of the raw kinetic data are made to take into account the global history of the growth curve and not only the difference between the values at the first and last time points. This method allows for the robust detection of both anti-infective and pro-infective molecules. Finally, our workflow comprises two secondary assays. A growth inhibition assay using fluorescent D. discoideum is used to determine an IC50 for negative effects on the host. This allows then to estimate a therapeutically window between effects on the intracellular bacteria and on its host. Yet, the data obtained for amoebae have to be validated for mammalian host cells, e.g. macrophages. The antibacterial assay performed on extracellular bacteria is used to quantify the difference in efficacy between the effect of the compound on extracellular versus intracellular bacteria, which will permit to identify compounds with most relevant anti-infective activities, being anti-virulence or host defence-boosting mode of action.

Overall, we detailed the establishment of an MTS pipeline for an ‘in vivo’ anti-tubercular screen allowing to test in moderate turnover time, 64 molecules per 96-well plates, with excellent sensitivity and good anti-mycobacterial specificity. Finally, we speculate that the use of amoebae hosts, which are natural vectors for many bacteria and have already proven powerful in the elucidation of mechanisms underlying host-pathogen relationships [47,79], also represent a worthy alternative model to contribute to hit and lead identification and drug development to fight tuberculosis.

**Materials and Methods**

**Bacteria and Cell Cultures**

_Acanthamoeba castellanii_ (ATCC 30234) was grown in PYG medium at 25°C as described (Moffat and Tompkins, 1992; Segal and Shuman, 1999) using proteose peptone (Becton Dickinson Biosciences) and yeast extract (Difco). The _D. discoideum_ strain expressing GFP-ABD [71] was grown in H15c medium at 32°C. Mycobacteria, the _M. marinum_ M-strain (wild-type), the _M. avium_ L1D mutant [65] and _M. smegmatis_ (generous gift from Gareth Griffiths) were cultured in Middlebrook 7H9 (Difco) supplemented with 10% OADC (Becton Dickinson), 5% glycerol and 0.2% Tween80 (Sigma Aldrich) at 32°C in shaking culture. _M. marinum_ and the _msp12::GFP_ plasmid were gifts from Dr. L. Ramakrishnan (Washington University, Seattle, USA). The _M. marinum_ strain expressing GFP in a constitutive manner was obtained by transformation with _mSP12::GFP_, and cultivated in the presence of 20 μg/ml kanamycin.

_Acanthamoeba_ Infection Assay

_A. castellanii_ were cultured in PYG medium in 10 cm Petri dishes at 25°C, and passaged the day prior to infection to reach 90% confluency. _M. marinum_ were cultivated in a shaking culture at 32°C to an OD600 of 0.8–1 in 7H9 medium. Mycobacteria were centrifuged onto a monolayer of _Acanthamoeba_ cells at an MOI of 10 to promote efficient and synchronous uptake. Centrifugation was performed at RT at 500 g for two periods of 10 min. After an additional 20–30 min incubation, extracellular bacteria were washed off with PYG and infected cells were resuspended in PYG containing 10 μM amikacin. 5 × 10^5 infected cells were transferred to each well of a 96-well plate (Cell Carrier, black, transparent bottom from Perkin-Elmer) with preplated compounds and controls. The course of infection at 25°C was monitored by measuring fluorescence in a plate reader (Synergy H1, BioTek) for 72 hours with time points taken every 3 hours. Time courses were plotted and data from all time points were used to determine the effect of compounds versus vehicle controls. To take into account possible autofluorescence of the compounds, RFU data of the first time point were subtracted from all time points. Cumulative curves were calculated. The activities of the compounds were determined by analysing maximum difference of compound cumulative curve to the 12–16 vehicle controls.

**Table 1.** Inhibitory concentrations 50% (IC50) values for _M. marinum_ growth in extracellular and intracellular conditions.

<table>
<thead>
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<th>antibiotic</th>
<th>extracellular IC50 (μM)</th>
<th>intracellular IC50 (μM)</th>
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<tr>
<td>streptomycin</td>
<td>0.1–1</td>
<td>3–7</td>
</tr>
<tr>
<td>fusidic acid</td>
<td>0.01–0.1</td>
<td>3–5</td>
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<tr>
<td>ethionamide</td>
<td>0.1–1</td>
<td>3–10</td>
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<tr>
<td>isoniazid</td>
<td>5–10</td>
<td>20–30</td>
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<tr>
<td>rifampin</td>
<td>0.1–0.5</td>
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<tr>
<td>rifabutin</td>
<td>2–3</td>
<td>1–3</td>
</tr>
<tr>
<td>ethambutol</td>
<td>5–10</td>
<td>5–10</td>
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<tr>
<td>levofloxacin</td>
<td>10–30</td>
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<tr>
<td>moxifloxacin</td>
<td>5–10</td>
<td>&gt;30</td>
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<tr>
<td>thioridazine</td>
<td>1–5</td>
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<td>PA-824</td>
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As presented in Figure 5C, GFP-expressing _M. marinum_ growth kinetics were measured in broth culture and during cellular infection in presence of antibiotics in a concentration range, and IC50 values were graphically determined from a series of experiments with similar outcome.

doi:10.1371/journal.pone.0087834.t001
Fluorescence Microscopy
Acanthamoeba cells were infected with GFP-expressing M. marinum as described above. Infected cells were monitored in 96-well plates (Cell Carrier, black, transparent bottom from PerkinElmer). Recordings were performed using a Leica LF6000LX microscope (100x 1.4 NA oil immersion objective).

Phase Contrast Microscopy
Acanthamoeba cells were infected with GFP-expressing M. marinum as described above. Infected cells were monitored in 96-well plates (Cell Carrier, black, transparent bottom from PerkinElmer). Recordings were performed using a CKX41 inverted microscope.

Antibiotic Activity Assay
10^8 GFP-ABD-expressing D. discoideum cells were transferred to each well of 96-well plates allowed to attach for 20–30 min. Cell growth at 25°C was monitored by measuring the GFP fluorescence in a fluorescent plate reader (Synergy H1, company) for 72 hours with a time point taken every 3 hours.

Growth Inhibition Assay
10^5 GFP-expressing M. marinum were transferred to each well of 96-well plates. Bacterial growth at 25°C was monitored by measuring the GFP fluorescence in a fluorescent plate reader (Synergy H1) for 72 hours with a time point taken every 3 hours.

Statistical Analysis
The Z factor was calculated using the means and standard deviations of both positive and negative controls (μp, σp and μn, σn). The following formula was applied: Z-factor = 1–3(σp+ σn)/ (σp+ σn) – μp– μn|.

Intracellular Replication of L. pneumophila
A. castellanii amoebae were cultured in PYG medium and passed the day prior to infection such that 2 x 10^5 cells were present in each well of a 96-well plate (Cell Carrier, black, transparent bottom from PerkinElmer). Cultures of L. pneumophila harbouring the GFP-producing plasmid pNT-28 were suspended from plates to a starting OD600 of 0.1 in AYE medium, and grown overnight on a rotating wheel at 37°C to an OD600 of 3. Bacteria were diluted in LoFlo medium (ForMedium) such that each well contained 8 x 10^5 bacteria, an MOI of 20. Infections were synchronised by centrifugation at 1500 rpm for 10 minutes. Infected cultures were incubated in a 30°C incubator and the GFP fluorescence was measured by a plate spectrophotometer at appropriate intervals (Optima Fluostar, BMG Labtech) [89]. Time courses were constructed and data from the point directly after entry up to stationary phase were used to determine the effect of compounds versus vehicle control.

Dictyostelium Growth on a Bacteria Lawn
Because D. discoideum cannot grow on lawns of virulent M. marinum, a specific growth assay was developed [89]. It consists in resuspending a pellet from one volume of centrifuged mid-log phase mycobacterial cultures in an equal volume of an overnight culture of K. pneumoniae diluted 10^6 fold. Then, 50 μl aliquots of the bacterial suspension were plated on 2 mL plugs of solid SM+Glucose agar medium in a 24-well plate format and left to dry for 2–3 hours. Finally, 1,000 D. discoideum cells were added on top of the bacterial lawn. Plates were incubated for 3–9 days at 25°C and the formation of phagocytic plaques was monitored and quantified.

Acknowledgments
We thank Ophelie Patthey for her technical help, and Dr Lalita Ramakrishnan for the msp12::GFP construct and the M. marinum M strain.

Author Contributions
Conceived and designed the experiments: SK VT CH HOS JM LS HH PC TS. Performed the experiments: SK VT CH HOS JM LS HH PC TS. Analyzed the data: SK VT CH HOS JM LS HH PC TS. Wrote the paper: SK VT HOS JM LS HH PC TS. Revised the manuscript: SK VT HOS JM LS HH PC TS.


Exploring anti-bacterial compounds against intracellular Legionella.

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VT’s contribution: Synergy Mx Monochromator-based multi-mode microplate reader (Biotek) maintenance, development and optimization of fluorescence-based D. discoideum infection assay used in Figures. 5D, 5E
Exploring Anti-Bacterial Compounds against Intracellular Legionella

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Abstract

Legionella pneumophila is a ubiquitous fresh-water bacterium which reproduces within its erstwhile predators, environmental amoeba, by subverting the normal pathway of phagocytosis and degradation. The molecular mechanisms which confer resistance to amoeba are apparently conserved and also allow replication within macrophages. Thus, L. pneumophila can act as an ‘accidental’ human pathogen and cause a severe pneumonia known as Legionnaires’ disease. The intracellular localisation of L. pneumophila protects it from some antibiotics, and this fact must be taken into account to develop new anti-bacterial compounds. In addition, the intracellular lifestyle of L. pneumophila may render the bacteria susceptible to compounds diminishing bacterial virulence and decreasing intracellular survival and replication of this pathogen. The development of a single infection cycle intracellular replication assay using GFP-producing L. pneumophila and Acanthamoeba castellanii amoeba is reported here. This fluorescence-based assay allows for continuous monitoring of intracellular replication rates, revealing the effect of bacterial gene deletions or drug treatment. To examine how perturbations of the host cell affect L. pneumophila replication, several known host-targeting compounds were tested, including modulators of cytoskeletal dynamics, vesicle scission and Ras GTPase localisation. Our results reveal a hitherto unrealized potential antibiotic property of the β-lactone-based Ras depalmitoylation inhibitor palmistatin M, but not the closely related inhibitor palmistatin B. Further characterisation indicated that this compound caused specific growth inhibition of Legionella and Mycobacterium species, suggesting that it may act on a common bacterial target.

Introduction

Legionella pneumophila is a ubiquitous environmental bacterium found in a wide range of fresh-water sources [1,2]. Bacteria within these environments suffer continuous predation from protozoa and as such have evolved a range of mechanisms to survive [3]. L. pneumophila produces a number of effector proteins which are injected into the host cell upon uptake. These effector proteins subvert the phagosome maturation process, instead promoting the formation of a biochemically distinct and replication-permissive “Legionella-containing vacuole” (LCV) [4,5,6]. The molecular processes by which this occurs are aimed at evolutionarily conserved molecular targets and are also utilized following uptake by human alveolar macrophages, thereby subverting a crucial cellular component of the innate immune system and allowing Legionella to act as an ‘accidental’ pathogen [7]. Through this process the inhalation of L. pneumophila can lead to the often fatal pneumonia known as Legionnaires’ disease.

The intracellular localization of L. pneumophila provides a challenge for targeting and eliminating the bacteria both in the environment and in patients. L. pneumophila within water systems such as air cooling ducts are commonly found residing within free-living amoeba, where they are protected from chemical decontamination [8,9]. Similarly, L. pneumophila within macrophages are more resistant to antibiotics, partly due to poor intracellular penetration of the compounds [10]. In addition, bacteria released from phagocytic cells are much more resistant to antibiotic treatment than their counterparts grown in broth alone [11].

Legionnaires’ disease is most commonly treated with combinations of macrolide and quinolone antibiotics, however...
Results

Single infection cycle intracellular replication of GFP-producing L. pneumophila

Testing anti-virulence, defence-boosting or antibiotic compounds requires a robust and complementary combination of infectious bacteria and replication-permissive host cells. L. pneumophila constitutively expressing GFP was utilised to allow non-invasive monitoring of intracellular replication. Protocols were developed for the infection of the environmental amoebae A. castellanii and D. discoideum, as well as the murine RAW 264.7 macrophage line. The ubiquitous amoeba A. castellanii, a common environmental host of L. pneumophila [1], was determined to be the most appropriate and robust host, with rapid, approximately 5-fold bacterial replication observed within 50 h (Figure 1A). The choice of LoFlo amoeba culture medium prevented L. pneumophila growth outside the cells while minimising auto-fluorescence. Multiplicities of infection (MOIs) above one ensured that the majority of the amoebae were infected at the beginning, and thus only a single round of intracellular replication was observed. Bacterial replication stopped after 50 to 100 h, depending on the MOI, suggesting an upper limit for the capacity of the amoeba to support the replication of intracellular bacteria (Figure 1A).

Intracellular replication was also examined within the commonly used, genetically tractable amoeba D. discoideum (Figure S1). The assays were performed at 25°C, as D. discoideum is unable to survive at higher temperatures for a prolonged time [22], which in turn led to a significantly slower (4-5 fold) replication over 150 h compared to growth in A. castellanii. For these reasons D. discoideum was deemed less suitable than A. castellanii for high- or medium-throughput compound screens. Further adaptation of the assay allowed the measurement of intracellular replication within murine RAW 264.7 macrophages (Figure 1B). Replication was less effective than in amoebae; with only a 2-3 fold increase observed after 50 h. Surprisingly, infection at low MOIs (2.5 or lower) resulted in an approximately 3-fold higher final bacterial load than that seen at higher MOIs. Owing to its robustness, speed and efficiency A. castellanii was utilised for initial compound testing, followed by validation using the more pathologically relevant macrophage cell line.

Having established the conditions for measuring intracellular growth in A. castellanii, the properties of known L. pneumophila deletion mutants were next examined (Figure 1C). Wild-type L. pneumophila replicated efficiently and reached a plateau phase approximately 50 h post-infection. In stark contrast, deletion of the Icm/Dot subunit IcmT, which impairs the T4SS and leaves replication stopped after 50 to 100 h, depending on the MOI, suggesting a common target or mode of action.

Inhibition of intracellular replication by low molecular weight compounds

The suitability of the L. pneumophila / A. castellanii assay for drug screening was further assessed by examining the effects of antibiotics. Infected cells were treated with gentamicin, ampicillin, kanamycin or neomycin at a range of concentrations, and replication was allowed to proceed until the vehicle control had entered stationary growth phase. Fluorescence data were normalized between 0 (background / no bacteria) and 1 (vehicle control), and dose-response curves were plotted (Figure 2). For comparison the dose-response inhibition for extracellular growth (i.e. in broth) for the same
antibiotics was determined and used to calculate the Cell Index, a measure of the degree of protection afforded to intracellular bacteria (Table 1). Intracellular bacteria were more resistant than extracellular to all antibiotics tested, an effect which was most apparent with ampicillin.

Next, the effects of compounds predicted to affect the host cell rather than the bacteria were examined. These compounds included taxol and nocodazole (targeting microtubules), latrunculin B (an actin polymerisation inhibitor), brefeldin A, dynasore and retro-1 (interfering with vesicle transport), as well as palmostatin M and B (blocking Ras signalling) (Figure 3). Of these, only latrunculin B, dynasore and palmostatin M caused significant reduction in either intra- or extracellular growth of *L. pneumophila* at 10 µM (Figure 3A). Taxol, nocodazole and brefeldin A inhibited intra- as well as extracellular replication of *L. pneumophila*, but only at high concentrations (Figure S2).

The dose-response curves of latrunculin B and dynasore were examined in more detail to identify IC$_{50}$ values (Figure 3B, C). Latrunculin B showed a low IC$_{50}$ (12 µM) for intracellular growth compared to dynasore (IC$_{50}$: 420 µM) (Table 2). Surprisingly, however, latrunculin B and in particular dynasore exhibited IC$_{50}$ values in the low micromolar range for extracellular growth and thus appeared to be effective antibiotics, rather than anti-virulence compounds. As observed with the classical antibiotics, intracellular bacteria were significantly less sensitive (> 3-fold) to these growth-inhibiting compounds.

**Palmostatin M selectively inhibits growth of *Legionella* and *Mycobacterium* species**

To characterize the apparent antibacterial activity of presumably host cell-active compounds, we focused on palmostatin M for further analysis. Palmostatin M inhibits the eukaryotic hydrolase enzymes APT1 and APT2 by blocking...
their Ras GTPase depalmitoylation activity and in turn altering Ras GTPase localization and activity [26,27]. The β-lactone Palmostatin M may also have off-target effects on bacterial serine hydrolases with a similar active site. Palmostatin M inhibited the intracellular growth of *L. pneumophila* in a dose-dependent manner in *A. castellanii* (Figure 4A) as well as in RAW 264.7 macrophages (Figure 4C). In contrast, the related β-lactone-based APT inhibitor palmostatin B did not inhibit intracellular replication in *A. castellanii* (Figure 4B) or in macrophages (Figure 4D). A secondary analysis for toxicity indicated that the palmostatin compounds did not affect the growth or viability of *A. castellanii* (Figure S2). Direct comparison revealed that extracellular *L. pneumophila* was much more sensitive to palmostatin M than intracellular bacteria (Figure 4E; Table 3). The extracellular replication of *L. pneumophila* was slightly inhibited by very high concentrations

**Table 1.** IC$_{50}$ values of antibiotics for intra- and extracellular *L. pneumophila*.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Intracellular</th>
<th>Extracellular</th>
<th>Cell index$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (µM)</td>
<td>95% CI (µM)</td>
<td>IC$_{50}$ (µM)</td>
</tr>
<tr>
<td>kanamycin</td>
<td>9.3</td>
<td>5-17</td>
<td>1.0</td>
</tr>
<tr>
<td>gentamicin</td>
<td>4.6</td>
<td>2.4-8.8</td>
<td>0.46</td>
</tr>
<tr>
<td>neomycin</td>
<td>11</td>
<td>3.5-35</td>
<td>3.8</td>
</tr>
<tr>
<td>ampicillin</td>
<td>N/C</td>
<td>N/C</td>
<td>31</td>
</tr>
</tbody>
</table>

$^1$ Intracellular versus extracellular

$^2$ N/C, not calculable

doi: 10.1371/journal.pone.0074813.t001

doi: 10.1371/journal.pone.0074813.g002

Figure 2. Sensitivity of intra- or extracellular *L. pneumophila* to antibiotics. A-D. *L. pneumophila* infecting *A. castellanii* (blue squares) or growing in AYE broth (black triangles) was treated with the antibiotics (A) kanamycin, (B) gentamicin, (C) neomycin or (D) ampicillin. Replication values were normalised between 0 (background / no bacteria) and 1 (replication of vehicle control), and dose-response curves were constructed from at least three independent experiments, with calculated lines of best fit.
Figure 3. Sensitivity of intra- or extracellular *L. pneumophila* to host-targeting compounds. A. The growth of *L. pneumophila* in the presence of various compounds (10 µM) was determined for both intracellular (*A. castellanii*) and extracellular replication. The graphs indicate the mean and 95% confidence intervals of OD$_{600}$ or fluorescence measurements normalised between 0 (background / no bacteria) and 1 (vehicle control) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (t-test) compared to DMSO control). B-C. Dose-response curves showing inhibition of intracellular (blue) and extracellular (black) replication by (B) latrunculin B, an inhibitor of actin polymerisation, or (C) dynasore, an inhibitor of dynamin-mediated vesicle scission. All graphs indicate the combined averages of at least 3 independent experiments.

doi: 10.1371/journal.pone.0074813.g003
of palmostatin B (Figure 4F). These experiments demonstrate that palmostatin M inhibits the growth of *L. pneumophila* in host cells as well as in broth, indicating an as-yet unrecognized potential as an antibiotic for this compound.

Next, species specificity was examined by determining the effect of 10 μM palmostatin M or palmostatin B on the extracellular growth of various *Legionella* species (Figure 5A). Palmostatin M prevented the growth of all *Legionella* species tested, whereas palmostatin B was ineffective against all species except *L. taurensis*. Further testing of palmostatin M or palmostatin B against a range of bacterial pathogens showed that their growth was unaffected by palmostatin M or palmostatin B (Figure 5B). Thus, palmostatin M selectively inhibits the growth of *Legionella* spp. in a group of various Gram-positive or Gram-negative, extra- or intracellular, vacuolar or cytoplasmic pathogens.

Although palmostatin M appeared specific to *Legionella*, another vacuolar pathogen is *Mycobacterium tuberculosis*, which - due to its biosafety level 3 requirements - was not originally tested. Interestingly, similar to *Legionella* spp. *Mycobacterium tuberculosis* grown in broth was efficiently inhibited by palmostatin M but not palmostatin B (Figure 5C). Since *M. tuberculosis* and *L. pneumophila* are both intracellular vacuolar pathogens, we determined whether palmostatin M could inhibit intracellular replication of a *Mycobacterium* spp. Due to a lack of suitable hosts for *M. tuberculosis* the closely related fish and opportunistic human pathogen *Mycobacterium marinum* was used, which replicates in *D. discoideum* [28,29] and *A. castellanii* amoebae (Kicka S. et al., manuscript in preparation). As with *M. tuberculosis*, palmostatin M but not palmostatin B efficiently inhibited the extracellular growth of *M. marinum* (Figure 5D), as well as the intracellular growth of *M. marinum* within *A. castellanii* (Figure 5E). Finally, dose-response curves indicated that, as with *L. pneumophila*, intracellular residence protected *M. marinum* against palmostatin M (Figure 5F). Thus, the β-lactone compound palmostatin M, but not the related compound palmostatin B, prevented both intra- and extracellular replication of *Legionella* and *Mycobacterium* species.

In attempts to identify the target of palmostatin M, the effect of palmostatin M and palmostatin B on the growth of a several defined deletion mutants of *L. pneumophila* was tested (Figure 6A, B). Similar to wild type *L. pneumophila*, palmostatin M but not palmostatin B inhibited growth of mutant strains lacking the T4SS subunit IcmT [23], the alternative sigma factor RpoS [24], the ClpP subunit of the ClpAP self-compartmentalizing protease [30], or approximately 18.5% of the genome [31]. The growth of these mutants was inhibited to a similar extent as wild-type *L. pneumophila*, indicating that the missing bacterial factors likely do not play a role regarding the mode of action of palmostatin M.

### Discussion

This study details the development of an assay to continuously monitor by fluorescence the intracellular replication of *L. pneumophila* within three phagocytic model host organisms, and shows how chemical inhibitors of growth can be characterised by this system. Previous attempts to develop new antibiotics via HTS have not had significant success [14], partly due to the difficulty of converting compounds which affect isolated bacterial factors into those with the same effect in live bacteria. The advantage of assaying compounds in a *L. pneumophila* / *A. castellanii* system is the ability to detect compounds which target the infection and intracellular replication process. However, this approach requires a robust secondary screen for compounds that simply kill the host cell. In this study the Alamar Blue viability assay was used to determine the cytotoxicity of compounds against *A. castellanii* host cells.

The complexity introduced by screening small molecule libraries against two interacting organisms raises the challenge to obtain reliable and reproducible results. Experimental values were used to calculate the Z-factor, a measure of the sensitivity and reliability of an assay [32]. The final calculated value of 0.61 indicates a satisfactorily robust assay, and is comparable to other screens that have been performed on intracellular pathogen replication [33]. In our assay, a higher variation between replicates was generally observed when compared to extracellular growth (e.g. Figure 3A), and thus it was necessary that all intracellular replication assays were performed in at least triplicate independent experiments. Thus the *L. pneumophila* / *A. castellanii* assay appears suitable for screening compound libraries at a medium-throughput scale (in the range of 10,000 compounds), however the greater variability and the challenges involved in scaling beyond 96-well plates are likely to prevent screening of larger libraries.

The process of intracellular replication was successfully quantitated in three host cells, *A. castellanii*, *D. discoideum* and RAW 264.7 macrophages. Whereas *A. castellanii* and *D. discoideum* behaved similarly, the macrophages showed higher final replication levels when infected at low initial MOIs. This might be due to an increased cytotoxicity of *L. pneumophila* against macrophages at higher MOIs, or due to a lower uptake efficiency allowing further replication of uninected macrophages during the assay; in effect working as a multiple rather than single-round assay. The requirement to incubate *D. discoideum* at the lower temperature of 25°C led to a significantly reduced rate of *L. pneumophila* replication, and thus, this amoeba was less suitable for screening than the more thermo-tolerant *A. castellanii*. *D. discoideum* is a commonly used model system for *L. pneumophila* infection, in large part due to the ease of genetic manipulation [34,35,36].

### Table 2. IC₅₀ values of host-targeting compounds for intra- and extracellular *L. pneumophila*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Intracellular IC₅₀ (µM)</th>
<th>95% CI (µM)</th>
<th>Extracellular IC₅₀ (µM)</th>
<th>95% CI (µM)</th>
<th>Cell index ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>latrunculin B</td>
<td>12</td>
<td>2.4-62</td>
<td>3.4</td>
<td>1.8-6.4</td>
<td>3.6</td>
</tr>
<tr>
<td>dynasore</td>
<td>420</td>
<td>0.9</td>
<td>0.6-1.5</td>
<td>470</td>
<td></td>
</tr>
</tbody>
</table>

¹ Intracellular versus extracellular

doi: 10.1371/journal.pone.0074813.t002
Figure 4. Sensitivity of intra- or extracellular *L. pneumophila* to palmostatin M and B. A-F. Time course of *L. pneumophila* infecting *A. castellanii* (A, B) or RAW 264.7 macrophages (C, D) in the presence of palmostatin M (A, C) or palmostatin B (B, D). Time courses are representative experiments showing mean and 95% confidence intervals of three wells. Dose-response curves of palmostatin M (E) or palmostatin B (F) indicate that palmostatin M inhibits replication of *L. pneumophila* in AYE (black triangles), *A. castellanii* (blue squares) and RAW 264.7 macrophages (red diamonds). Curves indicate averages and calculated curves for data from at least 3 independent experiments, normalised between 0 (no replication) and 1 (vehicle).

doi: 10.1371/journal.pone.0074813.g004
Therefore, while unsuitable for primary screening, *D. discoideum* allows later confirmation of suspected target proteins, for example those implicated in uptake, vesicle trafficking and replication of *L. pneumophila*, such as RtoA, PI3Ks, or Dd5P4 [36,37,38].

Initial validation using antibiotics showed that, as expected, the compounds prevented the extracellular growth of *L. pneumophila*. The dose-response curve was steeper for extracellular bacteria when compared to intracellular, whereas intracellular bacteria had a greater than 3-fold higher resistance against antibiotics. This suggests a greater overall efficacy of the antibiotics and/or a more uniformly sensitive population of extracellular bacteria. The findings are also in agreement with previous work, which has shown increased antibiotic resistance for intracellular bacteria [39] and for bacteria emerging from host cells [11].

Ampicillin did not affect intracellular replication, possibly due to expression of the *Legionella* β-lactamase gene, *foxA* [40], in combination with other genes differentially expressed in intracellular bacteria. Alternatively or additionally, access of the antibiotic to the phagocytic vacuole might be severely impaired, a mechanism that seems to apply to the human monocytic THP1 cell line [41]. In contrast, intracellular growth of *L. pneumophila* was inhibited by gentamicin, a nominally cell-membrane-impermeable antibiotic. This suggests that the interaction between the LCV and the outer milieu is more permissive than expected. The finding correlates with previous work, demonstrating that *Legionella*-infected human lung fibroblasts can be cured with gentamicin, thus indicating the accessibility of the pathogen vacuole to this antibiotic [42].

We also tested small molecules expected to affect bacteria-host cell interactions but not *L. pneumophila* directly, including compounds that target microtubules (taxol, nocodazole), actin polymerisation (latrunculin B), vesicle transport (brefeldin A [43], dynasore [44], retro-1 [45]) or Ras GTPase localisation (palmastatin M, an inhibitor of Ras depalmitoylation [26]). These compounds have been developed for mammalian cells, which may explain their moderate effect on replication within *A. castellanii*. Surprisingly, all molecules with an effect on *L. pneumophila* replication within amoebae were also able to prevent bacterial growth in broth, the most notable examples being dynasore and palmastatin M. Previous work has shown an antibacterial activity for minor structural variants of latrunculin B [46]; however the potent antibiotic activity of dynasore and palmastatin M has not been reported before. The antibiotic effects were also significantly more apparent in broth-grown *L. pneumophila* than intracellular bacteria, in line with previous observations that intracellular localization protects bacteria from otherwise toxic compounds [8,41].

Interestingly, our results indicate a specific antibacterial activity of palmastatin M, which inhibited the growth of all tested *Legionella* and *Mycobacterium* species, while having no effect on the growth rates of a wide range of other bacterial genera. A potential target protein of palmastatin M is the “self-compartmentalizing” bacterial protease ClpP, which has been shown to bind compounds with β-lactone rings such as palmastatin M in *Staphylococcus aureus* [47,48]. ClpP is not essential for *L. pneumophila*, but a deletion mutant strain exhibits slower growth rates and is unable to replicate in *A. castellanii* [30]. By contrast, the two homologues of ClpP in *M. tuberculosis* are essential for the survival of the bacteria, and have been validated as a target for acyldepsipeptides [49]. While *L. pneumophila* ClpP knockouts were equally affected by palmastatin M, it is possible that a homologous protein is the actual target. BLAST searches of the *L. pneumophila* genome revealed the existence of a homologue of human acyl-protein thioesterase 1, the original target of palmastatin M and B. This gene, *lpq0369*, is annotated as a hypothetical carboxylesterase/phospholipase (37% identity, 57% similarity), but as no analogue appears to exist in mycobacteria, it is unlikely to represent a common target in both *Legionella* and *Mycobacterium* spp. Further pull-down attempts with structural homologues of palmastatin M may provide more information.

Despite the current lack of knowledge regarding the palmastatin M target, this molecule represents a starting point for the development of potentially novel antibiotics. Inhibition of intracellular growth is a vital property for any compound developed against *Legionella* and *Mycobacterium* spp., both of which spend a significant and important part of their life cycle within eukaryotic cells [50,51]. The lack of broad spectrum antibiotic activity of palmastatin M does not preclude its development as a targeted antibiotic; indeed this may be preferable when coupled with improved diagnostic methods. However, one major pitfall of palmastatin M as an antibiotic is its previously established effect on APT1 (*IC₅₀ < 5 nM in biochemical assays [52]*) and thus on Ras localisation and signalling. Ras GTPase is involved in a wide range of cellular processes, including cell proliferation [53]. An *IC₅₀ < 5 nM* is about 1,000 × (*L. pneumophila*) or 100 × (*M. marinum*) lower than that observed to inhibit the replication of extracellular bacteria. Thus, careful screening of structural homologues is required to find compounds with specificity towards the bacterial targets rather than human acyl-protein thioesterases.

In summary, this study presents the development of a sensitive, discriminatory and continuous assay for the intracellular replication of *L. pneumophila* within the model host amoeba, *A. castellanii*. The assay was validated by determining the inhibitory concentrations required for antibiotics and a range of host-cell acting compounds. In this process an

**Table 3.** IC₅₀ values of palmastins for intra- and extracellular *L. pneumophila*.

<table>
<thead>
<tr>
<th></th>
<th>palmastin M</th>
<th>palmastin B</th>
<th>Cell index ¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (µM)</td>
<td>95% CI (µM)</td>
<td>IC₅₀ (µM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A. castellanii</td>
</tr>
<tr>
<td></td>
<td>9.8</td>
<td>1.9-50</td>
<td>18</td>
</tr>
<tr>
<td>RAW 264.7</td>
<td>14</td>
<td>5.9-32</td>
<td>N/C ²</td>
</tr>
<tr>
<td>Extracellular</td>
<td>1.2</td>
<td>1-1.5</td>
<td>72</td>
</tr>
</tbody>
</table>

¹ Intracellular versus extracellular

² N/C: not calculable
doi: 10.1371/journal.pone.0074813.t003
Figure 5. Species specificity of bacterial growth inhibition by palmostatin M.  A-E. Extracellular growth of (A) *Legionella* species (in AYE), (B) the bacterial species indicated (in LB), (C) *Mycobacterium tuberculosis* (in 7H9), and (D) *Mycobacterium marinum* (in 7H9) or (E) *M. marinum* infecting *A. castellanii* (MOI 10) was determined in the presence of 10 µM palmostatin M or palmostatin B. DMSO was used as a vehicle control. Palmostatin M specifically inhibited extracellular growth of the *Legionella* and *Mycobacterium* species tested. F. Dose response curve showing inhibition of intracellular (A. castellanii) (blue) and extracellular (black) replication of *M. marinum* by palmostatin M. Graphs indicates mean, error bars show 95% confidence intervals of at least 3 or 2 (*M. tuberculosis*) independent experiments (**p < 0.001 (t-test) compared to DMSO control).

doi: 10.1371/journal.pone.0074813.g005
Figure 6. Growth inhibition of *L. pneumophila* mutant strains by palmostatin M. The extracellular growth of *L. pneumophila* deletion mutants was determined in AYE medium in the presence of 10 µM palmostatin M (black bars), palmostatin B (grey), or DMSO (white). Palmostatin M equally affected *L. pneumophila* lacking (A) IcmT (component of Icm/Dot T4SS), RpoS (alternative sigma factor), or ClpP (catalytic subunit of ClpAP protease); as well as (B) 18% of the genome (ΔPenta). The OD₆₀₀ was measured after 24 h and normalized to the DMSO control. Graph indicates the mean and 95% confidence intervals of at least 3 experiments (**p < 0.001 (t-test) compared to DMSO control).

doi: 10.1371/journal.pone.0074813.g006
unexpected antibacterial property of the APT1 inhibitor palmoastatin M was discovered. The antibacterial activity of palmoastatin M appears to be specific to members of the genera *Legionella* and *Mycobacterium*. The assay thus shows promise for medium-throughput screening of libraries of small molecule compounds which affect bacterial intracellular replication.

**Materials and Methods**

**Bacterial strains, growth condition and reagents**

The bacterial strains used in this study are listed in Table S1. *L. pneumophila* JR32 ΔclpP was a kind gift of the Dr. Yong-jun Lu, Sun Yat Sen University, China [30]. The *L. pneumophila* pentuple deletion mutant was a kind gift of Dr. Ralph Isberg, Tufts University, USA [31]. The bacteria (except M. tuberculosis) were resuspended from plates in appropriate growth medium (ACES Yeast Extract (AYE) [54]) or Luria Broth (LB)) and diluted to a starting OD₆₀₀ of 0.01. Compounds were added to these cultures such that the maximal DMSO concentration was 0.1%. Cultures were grown overnight (or over several weeks in the case of *M. tuberculosis*) and the OD₆₀₀ measured. Data were normalized to DMSO controls and compounds compiled from the average value of at least 3 separate experiments.

Growth of *M. marinum* was assayed in BBL, Middlebrook 7H9 broth by seeding 1 × 10⁸ bacteria into a 96-well plate with or without compounds, and replication was followed for up to 48 h by monitoring the OD₆₀₀. *M. tuberculosis* suspensions were prepared at an OD₆₀₀ of 0.3 in BBL, Middlebrook 7H9 broth with glycerol (BD Bioscience). Triplicate cultures were prepared as a 500 µl suspension in 5 ml 7H9 broth in the presence of DMSO or 50 µM palmoastatin M. The cultures were incubated in tubes at 37°C and the OD₆₀₀ was measured every 2-3 days.

Reagents and compounds were from Sigma-Aldrich, if not indicated otherwise, whereas palmoastatin B and M were provided by Dr. Christian Hedberg, Max Planck Institute of Molecular Physiology, Dortmund.

**Intracellular replication of *L. pneumophila***

*A. castellanii* were cultured in PYG medium [21] and split the day prior to infection such that 2 × 10⁶ cells were present in each well of a 96-well plate (Cell Carrier, black, transparent bottom from PerkinElmer). Cultures of *L. pneumophila* harbouring the GFP-producing plasmid pNT-28 [19] were resuspended from plate to a starting OD₆₀₀ of 0.1 in AYE medium, and grown overnight on a rotating wheel at 37°C to an OD₆₀₀ of 3. Bacteria were diluted in LoFlo medium (ForMedium) such that each well contained 8 × 10⁴ bacteria (MOI 20). Infections were synchronised by centrifugation at 1500 rpm for 10 min. Compounds were added to at least triplicate wells during or after infection depending on the susceptibility time frame being assessed (see Data Analysis for more details). Infected cultures were incubated in a 30°C incubator, and the GFP fluorescence was measured by a plate spectrophotometer at appropriate intervals (Optima FluosStar, BMG Labtech). Time courses were constructed and data was used to determine the effect of compounds versus vehicle control.

Comparison of *L. pneumophila* deletion mutant strains was performed in a similar manner. To control for any changes in GFP fluorescence between strains the fluorescence/OD₆₀₀ was determined for each overnight culture and used to normalise later measurements. *D. discoideum* was cultivated in HL-5 medium, and infections were performed identically as *A. castellanii* with the sole change that infected amoebae were incubated at 25°C due to their thermosensitivity.

RAW 264.7 macrophages (ATCC: Tib-71, lab collection) were cultured in RPMI medium containing 5% FCS (Gibco). Cells were seeded at 8 × 10⁴ cells/well in a 96-well plate, to allow for the apparently lower infection efficiency. Infection was performed as with *A. castellanii*; however, bacteria and compounds were diluted in RPMI. The medium was changed 2 h post infection to ensure removal of non-phagocytosed bacteria.

**Intracellular replication of *M. marinum***

*A. castellanii* cells in PYG were allowed to settle on 10 cm dishes at 80% confluence. *M. marinum* (msp12::GFP, a kind gift from L. Ramakrishnan) were grown to a final OD₆₀₀ of 1 (5 × 10⁸ bacteria/ml) in flasks containing 7H9 medium supplemented with OADC (Oleic Acid Albumin Dextrose Complex), Tween 80, glycerol and kanamycin, as well as beads to assist growth. *M. marinum* were washed twice with PYG medium, and residual clumps were eliminated by passage through a 3 µm filter.

For the infection, the bacteria were homogeneously added onto 10 cm dishes containing approximately 2 × 10⁴ adherent *A. castellanii* (MOI 10). The dishes were centrifuged twice at 500 × g for 10 min and turned 180 degrees in-between the two centrifugation steps to avoid accumulation of cells and bacteria on one side of the dish. The cells were incubated at 25°C for 10-20 min, and excess extracellular bacteria were carefully removed by washing with fresh PYG. Amikacin (10 µM) was added at a concentration that completely inhibited extracellular bacterial growth without affecting intracellular growth. A 96-well plate suitable for fluorescence plate reading was filled with 100-200 µl of a suspension of infected cells; each well containing 2.5 × 10⁴ *A. castellanii* cells and incubated at 25°C. Fluorescence was recorded in a plate reader.

**Cytotoxicity Assay**

Cytotoxicity of compounds against *A. castellanii* was determined using Alamar Blue reagent (Life Technologies). To mimic the conditions found in the intracellular replication assay, 96-well plates were set up as previously described and uninfected triplicate wells were treated with compounds in 100 µl LoFlo media. Plates were incubated at 30°C for 24 h, after which 10 µl Alamar Blue reagent was added, and plates incubated for a further 3 h. The fluorescence at 595 nm was measured, and data normalized between 1 (treatment with LoFlo alone) and 0 (SDS, total lysis of the cells). Means from each individual experiment were then combined for analysis.

**Data analysis**

Data analysis was performed in Microsoft Excel and GraphPad Prism 5. To compare the effect of compound treatment on
intracellular replication, fluorescence values were taken from the first time point following entry to stationary phase. The results were then normalised such that media-only wells (no bacteria) were 0, whereas vehicle-treated wells were 1 (normal replication). The average of the replicate wells (minimum 3 per plate) was then plotted as dose-response curves, such that each individual point represented the average of a single experiment. Compound treatments were repeated a minimum of 3 times to control for the increased variability of bacteria-host cell interactions. Lines of best fit and associated IC50 values were calculated for each dose-response curve using the non-linear fit function of Prism 5 (log inhibitor versus response, variable slope). The Cell Index was calculated as the fold-increase in intracellular versus extracellular IC50.

Supporting Information

Figure S1. Replication of L. pneumophila within Dictyostelium discoideum. A-B. D. discoideum amoeba were infected in 96-well plates with GFP-producing (A) wild-type L. pneumophila at the MOIs indicated, or (B) wild-type L. pneumophila or mutants (MOI 10), and the progress of intracellular growth was followed by fluorescence measurement over 7 days using a microtiter plate reader. Intracellular replication of L. pneumophila in D. discoideum occurs over a longer time scale than that observed in A. castellanii, and the deletion mutants ΔcmT, ΔflaA or ΔropS also show replication defects in D. discoideum. C. Replication of L. pneumophila deletion mutants in A. castellanii was followed over the course of two days by CFU assay. Time course shows mean and standard deviation of the same representative experiment shown in Figure 1C and 1D.

References


4. Discussion
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To reach our goal of finding new compounds with antitubercular properties, we selected a strategy that involved amoebae and *M. marinum* as a host-pathogen model system. *M. marinum* was selected for its ability to generate TB-like pathogenesis at the same time maintaining relatively fast growth and low biohazard level for humans. As hosts we used two species of amoebae: *A. castellanii* and *D. discoideum*. *A. castellanii* was used for the primary screening since it is a large and efficient phagocyte that can survive at 37°C, a standard temperature of tuberculosis infection in humans. It also does not have complex developmental that makes it probably less sensitive to environmental stresses than *D. discoideum*. *D. discoideum* was used at later stages in growth assays and in the validation of the *A. castellanii* screen (see below). The usage of the *D. discoideum* model is especially beneficial for studies of the mechanism of action, due to the ease of genetic manipulation that this model provides.

We selected assays for quantification of bacterial numbers. CFU counting was excluded due to it being relatively low-throughput and low-consistency with clumping mycobacteria. Absorbance measurements were also rejected since they provide ambiguous signal with systems of complex optical properties such as an intracellular infection. Finally, we decided to use constitutively expressing GFP-reporters for calculation of bacterial loads. This approach allows efficient and robust detection of the changes in bacteria numbers. Our assay provides stable GFP signal proportional to the cell numbers. The measurements were performed during 60-72 hours.

Fluorescent GFP reporters were used in three types of assays: a bacterial growth assay, a host cell growth assay and infection assays. For bacterial growth assays, we measured changes of fluorescence intensity of GFP-expressing *M. marinum* in 7H9 medium. This assay allows detection of bactericidal and bacteriostatic activities although it cannot distinguish between them.

To detect actual killing events, secondary assays should be established, for example the assays that use short-lived fluorescent reporters or luciferase-based assays. In order to validate the *M. marinum* growth assay, we tested known antitubercular compounds. We tested the most potent first and second line drugs, such as isoniazid, rifampicin, ethambutol, pyrazinamide, levofloxacin, fusidic acid and others. The IC50 values obtained indicated acceptable levels of anti-infective detection. We could not obtain significant effects for pyrazinamide, AX20017 and levofloxacin, probably because of the short time of the measurements that cannot fully expose antitubercular properties of slow-acting drugs, such as
pyrazinamide. Assay specific causes and genetic differences may also be a factor. The predominantly successful validation indicates that our established assay can be used for detection of anti-mycobacterial activities.

Antimycobacterial activity in 7H9 broth is a crucial characteristic that is frequently used in antitubercular drug screenings. However, it is not sufficient for drug identification. Another more crucial factor is the activity of the compound in vivo, in particular its intracellular activity. Low intracellular activity results in failure to upgrade hits to the lead status. Abundance of false positive hits with high activity in bacterial broth and low intracellular activities is a common problem of many primary screens. In order to deal with this challenge, we designed our primary screen in vivo, in a host-pathogen system that includes A. castellanii as a host and GFP-expressing M. marinum as an intracellular pathogen. The D. discoideum infection assay was used at the stage of validation of primary hits. Assay establishment involved multiple rounds of optimization; suboptimal conditions were discarded. For example, we excluded to perform the infection assay in the chemically defined SIH medium that demonstrated unusual phenotype of fast mycobacterial release. In order to eliminate problems of extracellular bacteria we used the addition of 10 µM amikacin. As for the M. marinum growth assay, known anti-tubercular compounds were tested. Interestingly, we found out that the intracellular activity drops significantly for majority of the compounds. Apparently, the host demonstrates shielding properties due to low membrane permeability, host metabolism and/or activities of molecular pumps.

Host cell growth assays were used for estimation of cytotoxicity of the compounds. Similarly to M. marinum growth assay, host cell growth assay cannot distinguish between cytotoxic and growth inhibitory activities of the compounds. However, cytotoxicity can be easily monitored with the use of microscopic analyses. Two types of host cells were used: GFP-ABD-expressing D. discoideum cells for primary assessment of general cytotoxic activities by quantification of total fluorescent intensity in a platereader, and the microglial BV2 cells for validation of cytotoxicity at the mammalian cell level via visual inspection by high-content microscopy.

The infection assays developed here were used for the screening of three sets of chemical compounds with significantly different chemical and biological properties. These three libraries consist of the Sinergia library, compounds of the Malaria box and the GSK TB-set. All three libraries result from different approaches for compounds design and selection.

The Sinergia library is a set of 1260 highly chemically diverse compounds that were designed to target biochemical pathways involved in host-pathogen interactions. This library
was specifically built to cover maximal chemical and biological space. The library was screened in parallel in three in vivo screening systems that involved protozoan hosts funded by a Sinergia SNSF collaborative grant. Before the start of the project, the library had not been used in any other phenotypical screening.

In contrast to the Sinergia library, the GSK TB-set is a small collection of 180 antibiotic hits obtained in a screening that involved measurement of \textit{M. bovis BCG}/ \textit{M. tuberculosis H37Rv} growth in a broth. It was shown that all the GSK TB-set compounds display high antimycobacterial activities in 7H9 medium. Intracellular antimycobacterial activities of the GSK TB-set compounds had not been tested before this project.

Finally, the Malaria box library included 400 compounds with high activity against \textit{Plasmodium falciparum}. Usage of a library of hits identified using a genetically distant pathogen system allowed us to perform proof-of-concept studies and estimate possible specificity of the Sinergia library and the GSK TB-set.

Among the Sinergia library compounds, we distinguish compounds of various biological activities, including cytotoxicity, growth enhancement of \textit{M. marinum}, antibiotic activity in broth and intracellular antibacterial activities. In total, 22 anti-infectives and 44 pro-infectives were identified in the Sinergia library, which is a relatively high hit rate. Interestingly, many of the compounds with pro-infective activities showed to be host-specific and failed the validation step in microglial BV2 cells. Since identification of pro-infectives was not our primary goal, we decided to focus on anti-infectives. Nevertheless, investigation of pro-infectives could be a potentially interesting approach for the detection of defence pathways and molecular targets relevant to infection. Identification of 22 anti-infective compounds can be considered as a relatively high hit rate that confirms the specificity of the Sinergia library. The range of inhibition varied from 30% to 60%. Validation in microglial BV2 cells resulted in identification of 12 anti-infectives with inhibition of more than 20%. Additional SAR studies will hopefully help to refine the compounds to a lead level with higher antimycobacterial activities.

The Malaria box showed a very low hit rate. Only a single anti-infective was identified. This might have been expected, since it is a library of hits against another pathogen. Another possible explanation for the low hit rate is the lower concentration used in screening of the Malaria box compounds (5 µM instead of 30 µM for the Sinergia library screen, and 10 µM for the GSK TB-set).

On the other hand, the GSK TB-set understandably had high number of hits in our antibiotic assay, with a hit rate of 78%. This finding indicates high similarity between the \textit{M.}
*marinum*, BCG and *M. tuberculosis H37Rv* models. However, a significant number of GSK compounds showed decreased intracellular activity in BV2 cells. Some of the GSK compounds even failed to demonstrate *in vivo* activity at all. In order to find out whether decrease activity is host-specific, we compared the *A. castellanii* infection assay to the BV2 assay and identified generic shielding properties of *A. castellanii*. Results indicate that the GSK compounds display similar patterns of decreased intracellular *M. marinum* growth in both assays, although the *A. castellanii* assay is estimated to be at least 33% more stringent.

The influence of amikacin was also considered as a confounding factor. However, performing the BV2 assay in absence of amikacin demonstrated hit identification patterns with around 98% similarity with the assay with amikacin addition. This indicates that amikacin displays low synergistic activity in our assay and does not interfere gravely with hit identification.

To deepen the knowledge of mechanism of action of discovered hits, our group is planning to perform RNAseq experiments in infected *D. discoideum*. Comparative transcriptomic analyses will hopefully reveal the signalling pathways involved in anti-infective activity and help to establish target hypotheses. Analysis of transcription profiles may also explain host specificity of pro-infectives. Analysis of RNA expression of *D. discoideum* mutants will likely accelerate the discovery process. For example, changes in the infection phenotype in Atg1-null mutants in the presence of a selected compound will be a strong suggestion for the involvement of the autophagy pathway.

The shielding properties of alternative infection systems such as *A. castellanii* may also be beneficial. Increase of the stringency may cause the discovery of only the most potent hits. This potentially will allow to decrease the rate of false positive hits discovery, a well-known problem of today’s drug screening campaigns. On the other hand, higher-stringency may lead to emergence of false-negative hits which could be a significant problem. Our results suggest that the usage of protozoan hosts could be a promising alternative for drug screening campaigns. Our established amoebae-based infection screens demonstrate results comparable to screens in macrophage-related infection systems. The results of our *M. marinum* growth assay shares high level of similarity with the *M. bovis BCG/M. tuberculosis* antibiotic assays. Combination of high-throughput, low biosafety levels and ease of manipulation makes our approach a plausible alternative and an efficient tool for detection of specific *in vivo* antitubercular activities.
5. References


