Chapter 6

Testing tuberculosis drug efficacy in a zebrafish high-throughput translational medicine screen

ABSTRACT

The translational value of zebrafish high throughput screens can be improved when more knowledge is available on uptake characteristics of potential drugs. We investigated reference antibiotics and 15 preclinical compounds in a translational zebrafish-rodent screening system for tuberculosis. As a major advance we have developed a new tool for testing drug uptake in the zebrafish model. This is important because despite the many applications of assessing drug efficacy in zebrafish research, the current methods for measuring uptake using mass spectrometry do not take into account the possible adherence of drugs to the surface. Our approach combines nanoliter-sampling from the yolk using a micro-needle, followed by mass spectrometric analysis. As of to date no single physico-chemical property has been identified to accurately predict compound uptake; our method offers a great possibility to monitor how any novel compound behaves within the system. We have correlated the uptake data with high-throughput drug screening data from *M. marinum* infected zebrafish larvae. As a result, we present an improved zebrafish larvae drug screening platform which offers new insights into drug efficacy and identifies potential false negatives and drugs that are effective in zebrafish and rodents. Thereby we demonstrate that this improved zebrafish drug screening platform can complement conventional models of *in vivo M. tuberculosis* infected rodent assays. The detailed comparison of two vertebrate systems, fish and rodent, may give more predictive value for efficacy of drugs in humans.

Based on

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INTRODUCTION

Studies in zebrafish larvae are increasingly used for modeling human diseases with the expectation that they will be able to contribute towards bridging the gap between *in vitro* cell based assays and *in vivo* mammalian disease models[1]–[3]. Zebrafish larvae have become a popular vertebrate model due to: i) their anatomical, molecular and genetic similarity to humans, ii) the availability of a large genetic toolbox, iii) their easy and low cost maintenance, iv) the optical transparency of embryos and larvae enabling massive phenotype-based screens, v) very few ethical issues associated with the use of larvae up to the feeding stage and vi) the occurrence of many pathological processes resembling various human diseases. Amongst numerous other human diseases, tuberculosis (TB) has been modeled in zebrafish as a potential complement to the currently established *in vitro* and *in vivo* models proven to recapitulate the pathology of the human disease progression in zebrafish larvae[4]–[7]. In these systems efficacy of a drug can be accurately measured by its effect on the number of bacteria and their presence and survival inside immune cells[8]–[12]. As a result, zebrafish larvae have already yielded significant new insights into the understanding of the pathogenesis of TB in humans and contributed to the development of novel strategies for disease treatment[7]. Such a gain in understanding is very important since therapy of TB is becoming increasingly difficult with the emergence of new strains of bacteria resistant towards currently used antibiotic regimes (MDR, XDR, TDR)[13]–[17]. Although new drugs are being discovered the progress in FDA-approval of anti-TB medicines is very limited[18]. Moreover, there is also a lack of effective vaccination against TB pending new approaches and strategies that are under evaluation for further improvement[13]. Therefore the need for more efficient novel drugs, targeting either the pathogen or the host, remains as high and urgent as ever[17], [19]. This task can only be accomplished by bringing together academic institutes and pharmaceutical companies with the aim of quickly implementing novel tools that have the potential of accelerating the drug discovery process. As an example of this strategy, GlaxoSmithKline (GSK) recently made publically available the structure and anti-TB properties of a set of 177 potent anti-tubercular compounds, making samples available to the industrial and academic research communities to stimulate early-stage TB drug discovery activities[20].

Given its previously mentioned attributes, zebrafish larvae can be potentially used at various stages of the drug discovery process, ranging from target identification and lead optimization to preclinical and clinical development[21]. The easy accessibility to large larvae numbers and their small size makes this model particularly suitable for high-throughput *in vivo* screening of drugs added to the medium[1], [2], [8], [22]–[24], significantly reducing material requirements for testing. However, caution must be taken when interpreting the data in the absence of knowledge on the way of absorption of drugs in the tissues of zebrafish larvae [25].

While the pharmaceutical industry is increasingly gaining confidence in the use of zebrafish larvae in a number of toxicity studies[21], [26]–[28], progress in the application of zebrafish as a possible alternative or complement to traditional *in vitro* and *in vivo* drug efficacy models has been limited[11], [29], [30]. Despite the previously mentioned benefits, a possible explanation behind this lack of progress is the limited, or altogether missing understanding on the basic pharmacological parameters in zebrafish driving compound efficacy, namely uptake, distribution and metabolism[31].

Mass spectrometry (MS) is an analytical technique which is very suitable for the quantification and qualification of small molecules such as drugs and its metabolites. In the current state-of-the-art zebrafish assays, larvae are treated by adding the compound into their water container, and uptake is assessed by LC–MS analysis of whole larval lysates[28], [31]–[33].

Here we demonstrate that the whole larvae lysis method is not always suitable for determining the uptake of drugs. This is due to the capacity of some molecules to persistently adhere to the skin, even after thorough washing, resulting in artificially high background readouts and false positive results for drug uptake. To palliate this, we present an alternative drug uptake evaluation methodology in zebrafish larvae based on micro-needle sampling from the yolk followed by mass spectrometric analysis. Using this novel method two antituberculars in clinical use, rifampicin and moxifloxacin, and 15 preclinical lead compounds developed by GSK were examined. We correlate *in vivo* uptake levels with the efficacy of the compounds as measured in *M. marinum* and *M. tuberculosis in vitro* culture inhibition assays. Furthermore, the data obtained is also correlated with *in vivo* drug efficacy tests in a murine model of TB infection as well as with a high-throughput drug screening on *M. marinum* infected zebrafish (Figure 1). The results demonstrate the importance of standardized drug uptake studies in order to be able to understand and correlate the results obtained in zebrafish with other, commonly employed rodent efficacy models.

MATERIALS AND METHODS

Animal experiments ethic statement

All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals.

Zebrafish husbandry and compound treatment

Zebrafish of AB/TL wild-type strain were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). Embryos were collected from family crosses and grown at 28 °C in egg water (60 µg/mL Instant Ocean sea salts, Sera Marin) in dark. At 3 day post-fertilization (dpf) 20-30 larvae were transferred to small, 35 x 10 mm Petri dishes each containing 3-4 mL of egg water supplemented with the following compounds: rifampicin (RIF; Sigma-Aldrich) and moxifloxacin (MOX; Santa Cruz) each at 150 µM, and GSK compounds (GlaxoSmithKline Pharmaceuticals) at 10 µM concentration. For controls dimethyl sulfoxide (DMSO) at 0,1% was used. Larvae were exposed to the compounds for 17 and 40 hours. Controls were exposed for few seconds (T=0h). Compounds were administered separately to the larvae or in combinations. Compounds were refreshed every day.

Assessing compound quantities in zebrafish larvae with lysis method

Larvae were transferred to 2 mL micro-centrifuge tubes one by one; excessive compound was removed, washed three times briefly with 1 mL 50 % methanol and then prepared for lysis. Lysis was performed based on snap freezing in liquid nitrogen followed by sonication, as previously described[39].

Micro-needle sampling from the yolk of zebrafish larvae

Before taking samples, larvae were transferred to 2 mL micro-centrifuge tubes, and then washed three times briefly with 1 mL 50 % methanol one by one. After washing, one larva was placed on 1% agarose plate and excessive methanol was removed. Under stereomicroscope at 20 x magnification larvae were punctured in the yolk with a micro-needle (manually pulled borosilicate glass capillary, Harvard Apparatus) placed in the capillary holder of CellTram Oil micro-injector (Eppendorf) mounted onto a micro-manipulator workstation. Vacuum was generated manually via a rotating knob and the content of the yolk was extracted. When

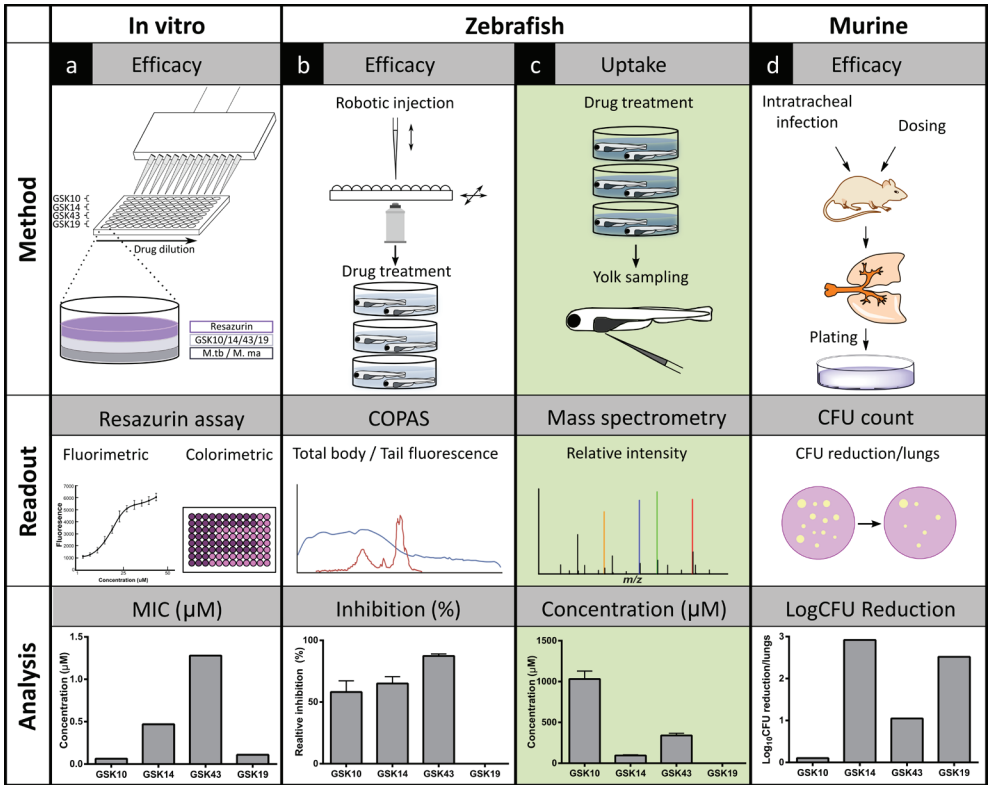

Figure 1: Scheme of drug efficacy methods: antibacterial activity and uptake assays in the zebrafish model integrated in conventional disease screening pipeline. Different models are used in our workflow to determine the efficacy of antitubercular compounds (from left to right): (a) Initially, the *in vitro* efficacy of the tested compounds are determined by their minimum inhibitory concentration (MIC) against *Mycobacterium marinum* and *Mycobacterium tuberculosis* cultures using fluorimetric and colorimetric read-outs from resazurin assay. Subsequently, biological validation is performed in *in vivo* models (b-d). (b) First, compound efficacy is screened in *M. marinum* infected zebrafish larvae. Embryos are robotically injected and following compound treatment the percentage of inhibition is determined using fluorescence read-out of COPAS system. (c) To unravel whether certain compounds fail to be active in zebrafish larvae due to lack of antibacterial activity or poor uptake, our micro-needle sampling method combined with mass spectrometry is used to assess uptake levels from samples of the yolk. (d) As gold standard in antitubercular drug development, compound efficacy is established by determining the rate of the colony forming units (CFU) reduction in the lungs of *M. tuberculosis* infected rodents. After setting (arbitrary) cut-offs in all models, compounds could be categorized into positive or negative groups. By the comparison of these groups along the pipeline, our improved zebrafish platform may give more predictive value for human efficacy of drugs.

the most of the content was removed from the yolk the suction was balanced and stopped. To recover the sample from the micro-needle into a micro-centrifuge tube a pressure was generated by turning the knob in the opposite direction.

Determination of the sample volume

Since individual larva can differ in their yolk size and volume; and the manually pulled micro-needles used for sampling were not identical in diameter, the sampled volume was not always the same per larva. In order to quantify the drugs in the sample, the volume has to be known. As a consequence, images of every sample were taken including the micro-needle containing

the sample. The volume of the samples was determined by modeling the needle as a cone and measuring the diameter and the length on the image, using the scale bar as a reference. Since the angle for the capillary holder was always adjusted in a 45° angle we also adjusted the model for this projection. This way the results were normalized with the according sample volume. Sample volumes were typically around 50 nL, varying between 20 - 200 nL.

Sample preparation
After recovering the sample from the micro-needle into the micro-centrifuge tube, 75% methanol including internal standards (IS) were added to the sample (rifabutin and levofloxacin as a IS for rifampicin and moxifloxacin respectively, and ampicillin as an IS for the GSK compounds). For the micro-needle samples an IS concentration of 20 nM was used. For the whole larvae samples the spiked IS resulted in a concentration of 250 nM. After vortexing for 2 minutes the precipitated proteins were spun down by centrifuging at 16.1 krcf for 5 minutes at 0°C. The supernatant was used for LC-MS analysis.

Mass Spectrometry
A Surveyor Plus UPLC system was used and 15 µL sample was injected on a Acquity C18 T3 column (2.1x100 mm, 1.8 µm). Mobile phase A was 99% H_2O and 1% acetonitrile with 0.3% formic acid. Mobile phase B was 90% acetonitrile and 10% H_2O with 0.3% formic acid and the flow rate was 500 µL/min. A LTQ Orbitrap XL (Thermo Fisher Scientific) was used for detection of the ions in positive electrospray mode. The electrospray voltage was 4.5 kV. The capillary temperature was 375°C and the capillary and tube lens voltage was 29 V and 120 V, respectively. The sheath gas flow rate and aux gas flow rate was 35 and 10 units, respectively. Resolution was set to 7500 in order to reduce the scan time and thus increase the number of data points. For the quantification of the samples an academic calibration curve was constructed within every batch.

***M. marinum* infections in zebrafish *in vivo* model**
Infection experiments were performed on zebrafish embryos at early developmental stages (up to 1,024-cell stage) injected with 40 CFU *Mycobacterium marinum M* strain into the yolk using automated robotic injection system as described in Carvalho *et al.*[8]. As a control an equal volume of carrier solution was injected. After injection, embryos were incubated at 280°C in egg water in Petri dish. Starting at 3 days post-fertilization (dpf) 200 larvae were treated with the following compounds till 5 dpf by adding the compounds into egg water: 200 µM rifampicin (Sigma-Aldrich), 10 µM GSK compounds and dimethyl sulfoxide (DMSO) (0,1%) as negative control. Water with compounds was refreshed once daily. Bacterial infection was quantified using Complex Object Parametric Analyzer and Sorter (COPAS) XL (Union Biometrica) as described previously[8]. Fluorescence in the posterior half of the larvae was determined using a custom perl script that analyses the COPAS extinction profile to determine the posterior half and then sums the fluorescent values of all data points for that half.

***M. tuberculosis* H37Rv inhibition assay**
The measurement of the minimum inhibitory concentration (MIC) was performed as described in Ballell *et al.*[20]. For each compounds measurements were performed in 96-well flat-bottom polystyrene micro-titer plates. Ten twofold drug dilutions in neat DMSO starting at 50 mM were performed. These drug solutions (5 µL) were added to 95 µL Middlebrook

7H9 medium (lines A–H, rows 1–10 of the plate layout). Isoniazid was used as a positive control; eight two-fold dilutions of isoniazid starting at 160 µg/mL were prepared, and this control curve (5 µL) was added to 95 µl Middlebrook 7H9 medium (row 11, lines A–H). Neat DMSO (5 µL) was added to row 12 (growth and blank controls). The inoculums was standardized to ~1x10^7 CFU/mL and diluted 1:100 in Middlebrook 7H9 broth (Middlebrook ADC enrichment, a dehydrated culture medium which supports growth of mycobacterial species, available from Becton–Dickinson, cat.# 211887), to produce the final inoculum of H37Rv strain (ATCC25618). This inoculum (100 µL) was added to the entire plate except G-12 and H-12 wells (blank controls). All plates were placed in a sealed box to prevent drying out of the peripheral wells and were incubated at 370°C without shaking for six days. A resazurin solution was prepared by dissolving one tablet of resazurin (VWR International Ltd., Resazurin Tablets for Milk Testing, cat.# 330884Y') in 30 mL sterile phosphate-buffered saline (PBS). Of this solution, 25 mL were added to each well. Fluorescence was measured (Spectramax M5, Molecular Devices; λ_{ex}=530 nm, λ_{em}=590 nm) after 48 h to determine the MIC value.

***M. marinum* inhibition assays**

The measurement of the minimum inhibitory concentration (MIC) was performed as described above in for the *M. tuberculosis* H37Rv inhibition assay with minor changes. Each compound measurement was performed in 96-well flat-bottom polystyrene microtiter plates. Ten two-fold drug dilutions in neat DMSO starting at 50 µM were performed. These drug solutions (2 µL) were added to 98 µL Middlebrook 7H9 medium (lines A–H, rows 1–10 of the plate layout). Isoniazid was used as a positive control; eight two-fold dilutions of isoniazid starting at 100 µM were prepared, and this control curve (2 µL) was added to 98 µL Middlebrook 7H9 medium (row 11, lines A–H). Neat DMSO (2 µL) was added to row 12 (growth and blank controls). The inoculum was standardized to ~2x10^5 CFU/mL and diluted 1:1 in Middlebrook 7H9 broth (Middlebrook ADC enrichment, a dehydrated culture medium which supports growth of mycobacterial species, available from Becton–Dickinson, cat.# 211887), to produce the final inoculum. This inoculum (100 µL) was added to the entire plate except C-12 and D-12 wells (blank controls). All plates were placed in a sealed box to prevent drying out of the peripheral wells and were incubated at 280°C without shaking for three days. A resazurin solution was prepared by dissolving one tablet of resazurin (VWR International Ltd., Resazurin Tablets for Milk Testing, cat.# 330884Y') in 30 mL sterile phosphate-buffered saline (PBS). Of this solution, 25 µL were added to each well. Fluorescence was measured (Tecan infinite 200Pro; λ_{ex}=535 nm, λ_{em}=590 nm) after 48 h to determine the MIC value.

***M. tuberculosis* infections in mouse *in vivo* model**

Infections of mice with *Mycobacterium tuberculosis* was initiated by nonsurgical intratracheal instillation of *M. tuberculosis* H37Rv as previous described by Rullas *et al.*[29]. In brief, 8- to 10-week-old female mice were anesthetized with 3% isoflurane and intubated with a metal probe (catalog number 27134; Unimed SA, Lausanne, Switzerland). The inoculum (10^5 CFU/mouse suspended in 50 µL of phosphate-buffered saline) was put into the probe and delivered through forced inhalation with a syringe. Treatment was started 24 hours after infection, to allow for phagocytosis of instilled bacteria, and lasted up to 7 days post infection. Finally, an additional 24 h was allowed for clearance of compounds before organ harvesting. To measure infection burden in lungs, all lobes were aseptically removed and homogenized. The homogenates were supplemented with 5% glycerol and stored frozen (-80 °C) until plating. After 14 days of culture, colonies were counted using an automatic colony counter (aCOLyte-Su-

percount; Synoptics Ltd., Cambridge, United Kingdom) and confirmed by visual inspection to correct potential misreadings. Bacterial growth of about 2 logs over the initial inoculum was determined to be a level that would provide enough dynamic range to detect statistically significant growth inhibition.

Statistical Analysis

COPAS data were analysed (Prism 4.0, GraphPad Software) using nonparametric, two-tailed Wilcoxon signed-rank test, and the uptake data were analysed using unpaired, two-tailed t-test. P values shown are: *P<0.05, **P<0.01, and ***P<0.001.

RESULTS

Assessing rifampicin and moxifloxacin quantities in zebrafish by lysis of whole larvae

We have recently reported an automated high-throughput drug screening protocol using *Mycobacterium marinum* infected zebrafish larvae[8]. To better understand the efficacy of antituberculosis compounds used in this test system, we performed uptake experiments using mass spectrometry. This improved zebrafish drug screening platform could then be integrated into the conventional disease screening pipeline and give more predictive value for human efficacy of antitubercular compounds (Figure 1).

To assess the quantity of drugs present in zebrafish, larvae were exposed to rifampicin, a first-line anti-TB drug used as a positive control in our drug screening experiments[8], [22], as well as to moxifloxacin, a gyrase inhibitor currently under exploration for novel antitubercular regime development to shorten the duration of TB treatment[34].

First we measured the quantities of the aforementioned compounds using the previously described whole larvae lysis method followed by LC-MS analysis[28]. After different exposure times (17 and 40 hours) larvae were washed with 50% methanol and lysed individually to determine drug concentration using LC-MS (Figure 2a). A steady increase was detectable in the level of moxifloxacin during the treatment period from 3.1 pmol/larva at 17 hours of exposure and increasing further to 13.6 pmol/larva at 40 hours of exposure. On the other hand, rifampicin seemed to have reached a steady state level already at 17 hours of exposure with 6.2 pmol/larva and 6.6 pmol/larva at the 40 hour exposure time. As a control we quickly exposed the larvae to the drugs for only a few seconds, then washed them immediately with 50% methanol and lysed them for analysis (T=0 h). Surprisingly, the presence of both drugs was detected even after these few seconds of exposure, at which time rifampicin was measured at 4.6 pmol/larva and moxifloxacin at 0.8 pmol/larva. Washing after drug exposure was also performed using other solvents (embryo medium, 5% and 50% ethanol), which resulted in the same quantitities as were detected after 50% methanol wash (data not shown). Detection of rifampicin and moxifloxacin in the T=0 h control lysates was presumably due to the tendency of these drugs to adhere to the skin of the larvae. These results suggest that the whole larvae lysis method is not suitable for monitoring drug uptake.

Determining uptake of rifampicin and moxifloxacin using micro-needle sampling

Since the larvae lysis method cannot be used for correctly monitoring the uptake of certain drugs, we aimed to overcome this issue by taking samples (in the nanoliter range) from the yolk of zebrafish larvae using a micro-needle attached to an Eppendorf CellTram Oil. As detailed in the material and methods section we also have developed a method to accurately determine the sample volume needed for quantitative mass spectrometric analysis. This method was used to analyze samples in the same time-course experiment as applied in the lysis method (17 and 40 hours of exposure). We observed a gradual increase in the uptake

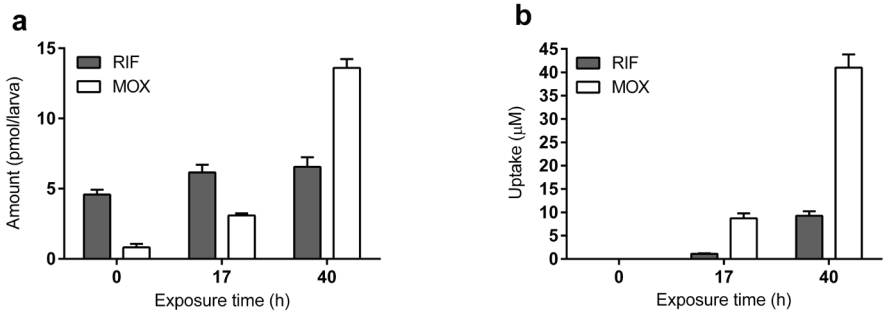

Figure 2: Rifampicin and moxifloxacin quantities measured by (a) whole zebrafish larvae lysis method and (b) micro-needle sampling method from the yolk. Three day-old zebrafish larvae were exposed to the combination of rifampicin (RIF; grey bars) and moxifloxacin (MOX; white bars) dosed at a 150 µM concentration for 0, 17 and 40 hours (n = 10). Note that as a control at 0 time point (T=0 h) the zebrafish larvae were exposed to the compounds (at the same dose) for only few seconds. This short exposure was followed by three consecutive washing steps with 50% methanol. In the read-out, (a) after lysis of the whole larvae the presence of compounds is detected even at T=0 h, however (b) via our micro-needle method the compounds are undetectable in the yolk at T=0h. All data are expressed as the mean ± S.E.M.

level of both rifampicin (17 hours of exposure at 1.1 µM, 40 hours of exposure at 9.2 µM) and moxifloxacin (17 hours of exposure at 8.7 µM, 40 hours of exposure at 41 µM) (Figure 2b). Neither rifampicin nor moxifloxacin was detected at the control time point (T=0 h), indicating that our new method eliminates the issue of drug adherence. The results show that our method can measure drug uptake with high accuracy and sensitivity from samples in the nanoliter range and is also suitable for compounds that persistently stick to the surface of larvae such as rifampicin.in the nanoliter range and is also suitable for compounds that persistently stick to the surface of larvae such as rifampicin.

Uptake of preclinical anti-tuberculosis compounds and correlation with their efficacy on bacterial burden in infected zebrafish larvae

In order to correlate the uptake of a drug with its *in vivo* effect on microbial infection using drugs that were not previously tested in our zebrafish model, we performed an anti-TB drug screening on *M. marinum* infected zebrafish larvae using 15 preclinical compounds provided by GSK. These compounds were pre-screened *in vitro* for their antibacterial activity on *M. tuberculosis* and *M. marinum* cultures showing a large gradient of activities (Supporting Table 1). For the *in vivo* test, zebrafish embryos were robotically injected with fluorescently labeled bacteria at early stages of development and hatched larvae were treated with compounds from 3 days post-infection (dpi) following the protocol as published by Carvalho *et al.*[8]. The antibacterial efficacy of the compounds was assessed by monitoring the fluorescence intensity correlating with bacterial burden present either in the total body or only in the tail region of 5 day-old zebrafish larvae using COPAS analysis. The efficacy was expressed as percentage of inhibition relative to DMSO treated control groups (Figure 3). The tail region is representative of a part of the body where mycobacteria are mainly enclosed by immune cells leading to granulomas and therefore presents a good measure for disseminated disease[8], [35]. The tail measurements compared to the total body are an indicator for potential problems with drug distribution. Compared to the control we identified compounds reducing bacterial load significantly and others that had moderate or no significant effect on infection (Figure 3). The results show that at least 3 compounds were significantly active on reducing bacterial burden both in the total body and the tail region. In order to better understand the reason for a com-

pound failing to lower bacterial load in the zebrafish, we determined GSK compound levels in samples taken from yolk of larvae at 5 dpf after 40 hours treatment (Figure 3a). The comparison revealed a correlation between the uptake and the antibacterial efficacy of compounds both in the total body and the tail region (Figure 3a, b, c).

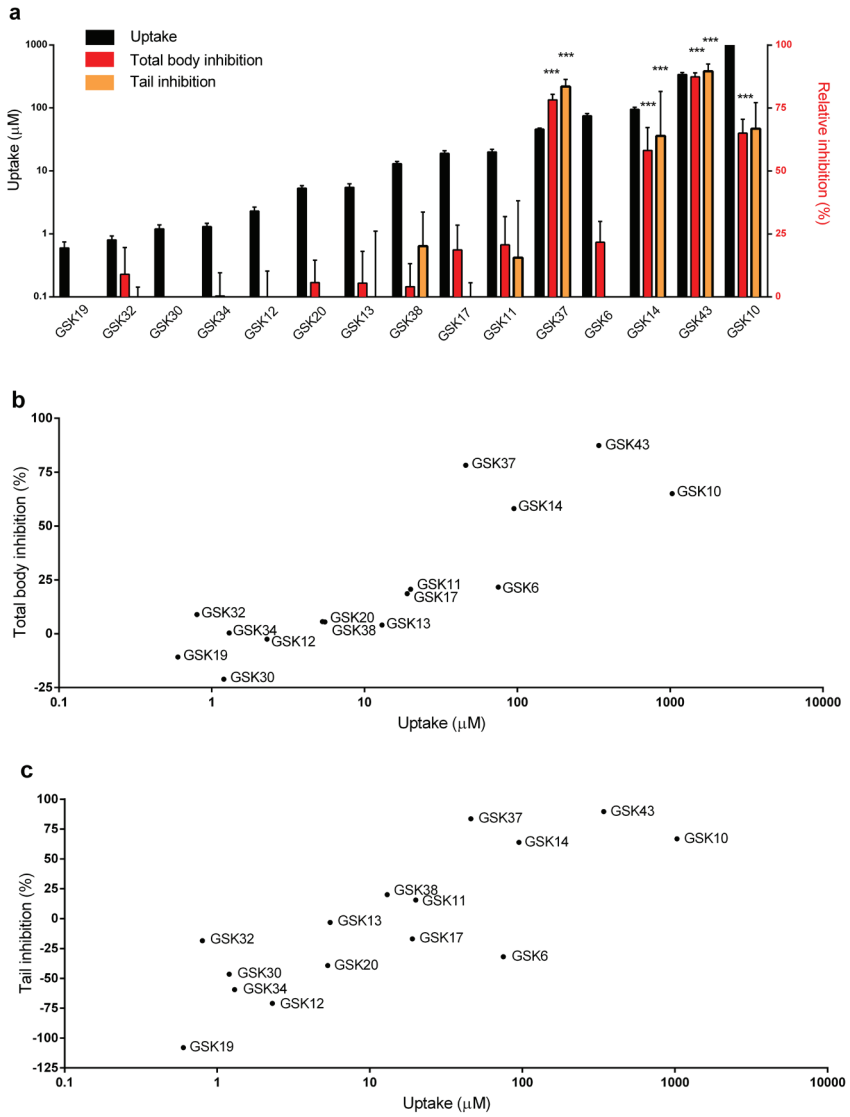

Figure 3: Correlation between the uptake level of preclinical antitubercular compounds and their efficacy in *M. marinum* zebrafish infection model. (a) Uptake levels of 15 preclinical antitubercular GSK compounds were measured from samples taken from the yolk of 5 day old zebrafish larvae after 40 hours exposure at 10 µM concentration (black bars) (n=10). Efficacy of the compounds was assessed by monitoring fluorescent bacterial burden in the total body (red bars) or tail region (orange bars) of 5 dpi larvae using the COPAS system after 40 hours treatment (n=200). Efficacy is expressed as percentage of inhibition relative to DMSO treated control groups. The bar graphs depict the correlation between the uptake and the relative inhibition, both in the total body and the tail region. Significance in inhibition is indicated with asterisks (***P<0.001). (b, c) Correlation between the efficacy (b) in the total body or (c) in the tail and the uptake of the GSK compounds.

Correlation between drug uptake levels and physico-chemical properties

It is important to determine if the uptake level of a compound can be correlated with its physico-chemical properties; if such a relationship was identified it would allow effective prediction of the uptake of compounds in this model. We compared a number of common metrics used in predictive strategies to the uptake levels measured in our assays. In our case no relationship was found for the majority of the metrics examined (molecular weight, molecular volume, number of rotatable bonds, hydrogen bond acceptors/donors, number of sp3 carbons, number of aromatic rings, number of Lipinski H-bond acceptors/donors, and polar surface area; see Supporting Figure 2). One metric did show a weak relationship with the measured uptake, chromlogD – which represents the lipophilicity of a compound (Figure 4). This, experimentally determined property, is the chromatographic hydrophobicity index (CHI)[36], a retention time of the compound at pH 7.4 on a fast gradient reverse phase HPLC column, modified by a conversion factor which has been shown to be more accurate than classical OW partitioning[37]. Only a weak correlation was identified between high chromlogD (above 3.5) and uptake. This is in line with other findings and confirms that it is hard to accurately predict uptake of compounds solely based on their physico-chemical properties such as lipophilicity[21], [31]. Our micro-needle sampling method gives valuable additional information on transportability of particular drugs through epithelial layers and could lead to better theoretical models for use in combination with physico-chemical predictions.

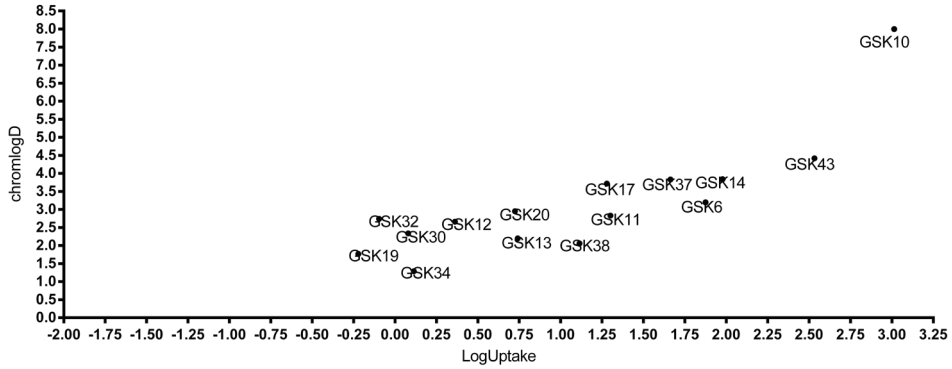

Figure 4: Correlation between uptake levels and compound hydrophobicity. ChromlogD is the representation of the hydrophobicity and therefore the solubility/ lipophilicity of a compound. To examine the relationship between ChromlogD of the compounds and their uptake ChromlogD is plotted against uptake levels measured in 5 day old zebrafish larvae after 40 hours exposure. A weak correlation is observed between high chromlogD (above 3.5) and uptake.

Comparison of uptake levels with *in vitro* and *in vivo* antibacterial efficacy models used in our pipeline of anti-TB drug discovery

We aimed to elucidate if the uptake and antibacterial efficacy results obtained from the zebrafish model translate to other models commonly used in the drug development pipeline. For this reason, we first compared zebrafish data to *in vitro M. marinum* antibacterial efficacy models.

To determine if there is correlation between uptake and the antibacterial activities, first we normalized the data for the uptake with the minimum inhibitory concentration (MIC) of compounds against *M. marinum* (Figure 5). This Log(uptake/MIC) ratio identifies which compounds are present in the larvae at concentrations in excess of their MIC values. Using this data a plot of the Log(uptake/MIC) against the observed percentage of inhibition in the zebrafish assay was produced (Figure 5). As expected, none of the 3 compounds of which

the Log(uptake/MIC) was below zero significantly reduced the bacterial burden in the entire body in zebrafish (GSK12, GSK13, GSK19). However, from the 12 compounds, which were present in concentrations well above their MIC values, only 4 were active (indicated with green) (GSK 10, GSK14, GSK37, GSK43).

Furthermore, we extended our comparison with *in vitro M. tuberculosis* antibacterial efficacy data as well as with *in vivo M. tuberculosis* infected mouse efficacy data, the latter being a gold standard in anti-tuberculosis drug development (Figure1). In the acute mouse model the inactive compounds are defined as compounds that produced less than a 2 log reduction in CFU count[29] (Supporting Table 2). Conversely, active compounds are those that produced a greater than 2 log reduction in the CFU count in the acute mouse model. Of the 4 active compounds identified via the zebrafish screening only 2 (GSK14, GSK37) are considered as active in mouse experiments, whereas the other 2 compounds (GSK10, GSK43) were inactive in the mouse model. Of the 11 compounds detected as inactive in zebrafish assay 6 proved to be inactive in mouse as well (GSK6, GSK12, GSK13, GSK30, GSK34, GSK38) showing that both *in vivo* models yield very different results from the *in vitro* MIC data.

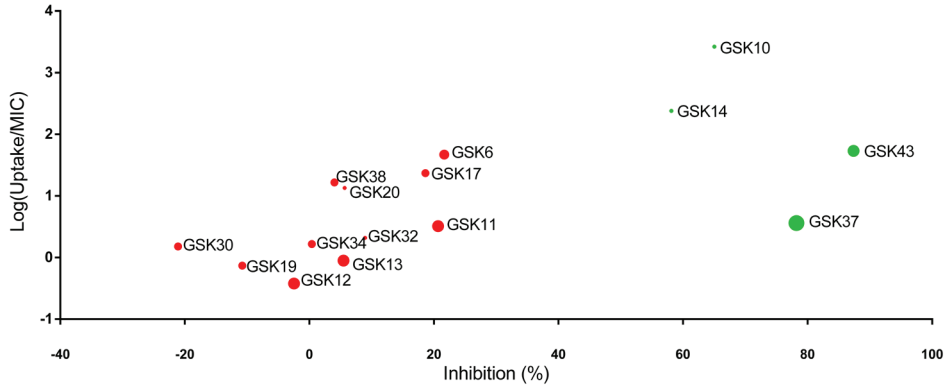

Figure 5: Correlation between uptake and in vitro minimum inhibitory concentration and *in vivo* zebrafish antibacterial activities of compounds. The measured uptake data in zebrafish larvae is normalized with *in vitro* minimum inhibitory concentration (MIC) of compounds against *M. marinum* (Log(uptake/MIC)). This ratio is plotted against the observed percentage of inhibition in the zebrafish *M. marinum* infection assay. Size of the circles indicates their MIC values. Green colour represents compounds significantly reducing bacterial burden in zebrafish infection model, considered as active compounds, while red colour indicates inactive compounds.

DISCUSSION

Zebrafish larval screening systems have increasingly been used for testing the effects of drugs. Especially, in the context of drug screening against tuberculosis, current methodologies based on bacterial load reduction are accurate and can be automated. In the case of antibiotic screens this has led to valuable information on the efficacy of existing and new antibiotics using *M. marinum* infection as a test system[8], [11], [12], [38]. However, the reasons behind the failure of some compounds to lower bacterial load in *M. marinum* infected zebrafish larvae were unclear and we wanted to test whether this was caused by poor compound uptake. To date, the mass spectrometric methods currently in use fail to correctly monitor uptake levels in zebrafish. In order to address this problem, we decided to explore an alternative methodology that could measure uptake of compounds from the yolk of zebrafish larvae by micro-needle sampling followed by mass spectrometric analysis. The results show that micro-needle sampling of volumes in the nanoliter range from the yolk allows accurate and sensitive analysis of uptake of drugs by individual larvae.

Our method is based on the assumption that taking samples from the yolk is relevant for predicting limitations of drug efficacy in the rest of the body of zebrafish larvae. This assumption is supported by measured quantities in the yolk and whole larvae lysis experiments for moxifloxacin (Supporting Figure 1). We have compared antimicrobial efficacy in the entire body with the tail region in order to check whether spreading of the tested drugs through the body could present a problem (Figure 3). These results do not show any indication that the effect of the 15 tested compounds is limited by distribution through the body and therefore indicate that the yolk is a representative location for measuring drug uptake.

Our results show that there is a high variety of uptake of the various tested compounds. As expected, only compounds that reach uptake levels that are greater than their MIC are active in our antimicrobial test system. In some cases uptake shows to be a limiting factor for reaching the MIC as determined in vitro (Figure 5). The fact that this outcome could not be predicted solely based on physico-chemical properties like hydrophobicity or mass shows the importance of our test system. At least in one case (compound GSK19) it is clear that the difference with the effect in the rodent test system can be explained by problems with uptake by the zebrafish larva (Figure 1). By explaining false negative test results from large screens, our microsampling method opens new possibilities for drug screening using zebrafish larvae in a preclinical test set up. In the future our screening pipeline can also be the basis for in depth studies of the mechanisms behind this and other false negative test results in zebrafish. It is also interesting to study why some drugs that did show high efficacy in the zebrafish larvae model were inactive in the mouse model. This could be examined for instance by testing other drug administration methods, e.g. drugs can be injected directly inside the body or added to the food instead of the medium. This can show whether the negative results in the rodent test model are caused by degradation of drugs added to the food in the acidic environment of the stomach.

In addition to highlighting the importance of identifying potential false negative and positive test results, our results show that some drugs that come out as most positive in the combined uptake and efficacy zebrafish test system are also active in the *in vivo* rodent model. This is of significance since zebrafish larvae do represent a very different test system as compared to rodents for instance with respect to (1) method of application (in the medium versus application in the food in the rodent system), (2) metabolic rate of these test system (where zebrafish present a metabolic rate that is much slower than rodents), and (3) different levels of genetic polymorphisms (the zebrafish strains are wild types with very high number of polymorphisms whereas the mouse strains are highly inbred and devoid of polymorphisms). We therefore believe that the zebrafish model provides added value to the rodent system since, in comparisons with the human population, test models with a larger number of polymorphisms and a lower metabolic rate are of relevance. In a broader perspective the observation that compounds that are active in two highly different test systems (in this case a fish versus a rodent) could provide additional confidence that the compound will also provide high efficacy in human subjects with varying background genotypes. Some of the compounds tested in this study (such as GSK14, which was shown to have good uptake and high efficacy in the zebrafish system) are members of series of compounds which are currently progressing to extensive medicinal chemistry programs that hopefully lead to clinical applications in the near future.

We therefore anticipate that the novel uptake measurement technique presented here makes important inroads to our understanding of zebrafish infection model data and opens up new opportunities for zebrafish larval systems to be more widely employed in the field of pharmacology. It should be of notice that our uptake method is also highly applicable for toxicity

testing in zebrafish larvae testing and in this case it will also be of great added value to rodent toxicity test systems. We hope that our results will stimulate future detailed follow-up pharmacokinetic studies in this model system. In conclusion, our results are a good starting point to undertake more detailed PK/PD studies together with studies of reference compounds that eventually can lead to full acceptance of the zebrafish as a model in pharmacological screening pipelines in industry.

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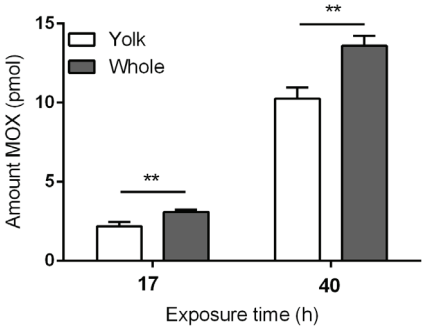
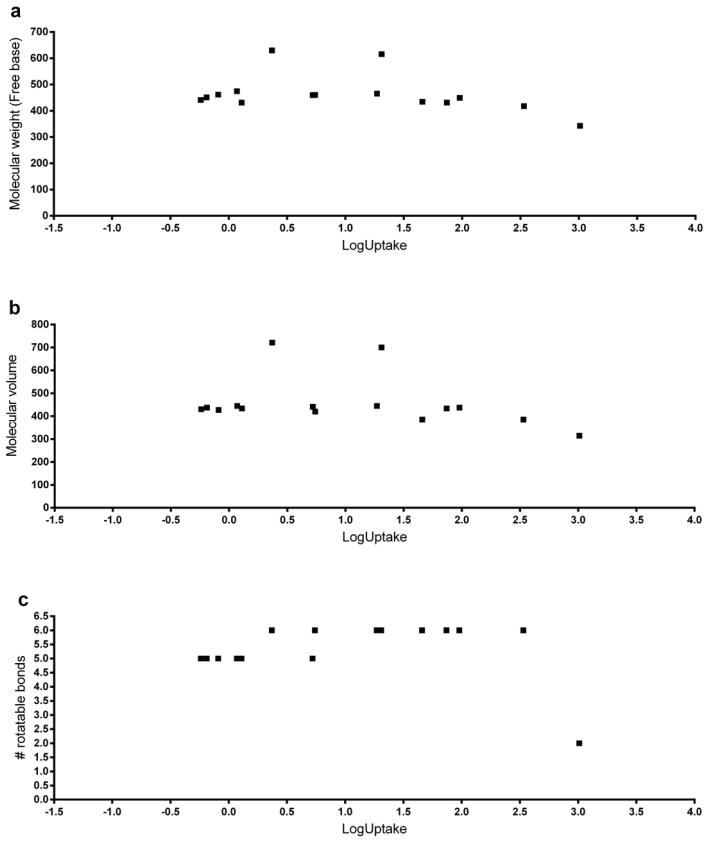
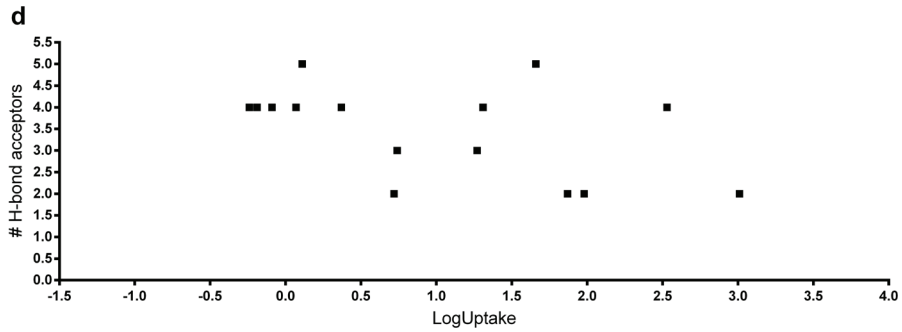
SUPPORTING INFORMATION



Supporting Figure 1: Comparison of the amount of moxifloxacin measured in the samples from the yolk and lysates of whole zebrafish larvae. Level of moxifloxacin was measured after 17 (4 day old larvae) and 40 hours (5 day old larvae) of drug exposure using samples from the yolk (white bars) and from the lysates of whole larvae (grey bars) (n=10). Data are expressed as the mean ± S.E.M. Significance is indicated with asterisks (**P<0.01).

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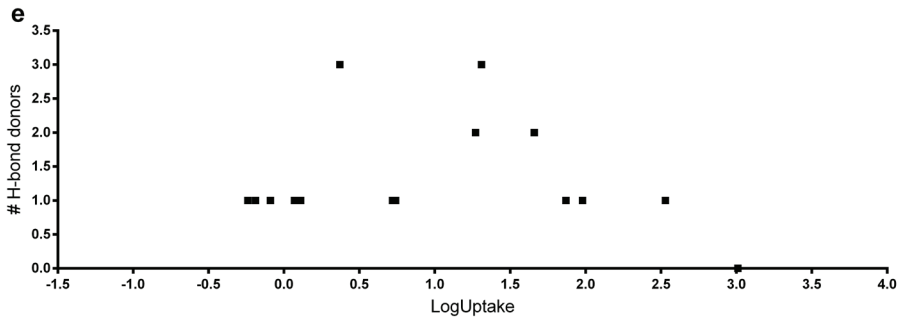
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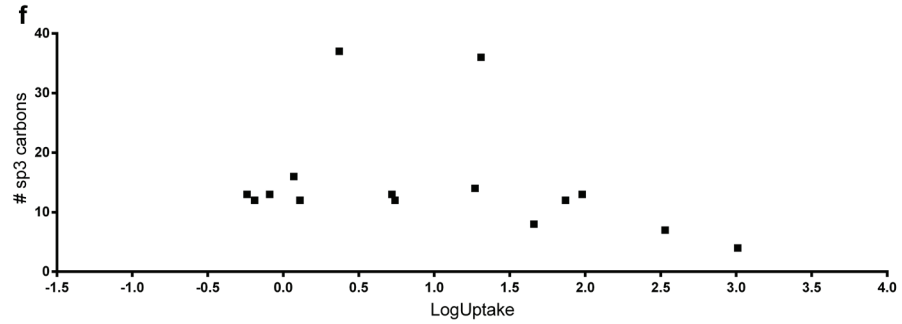
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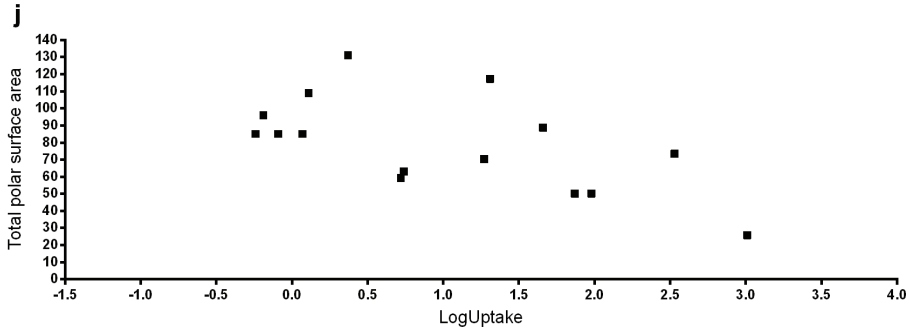
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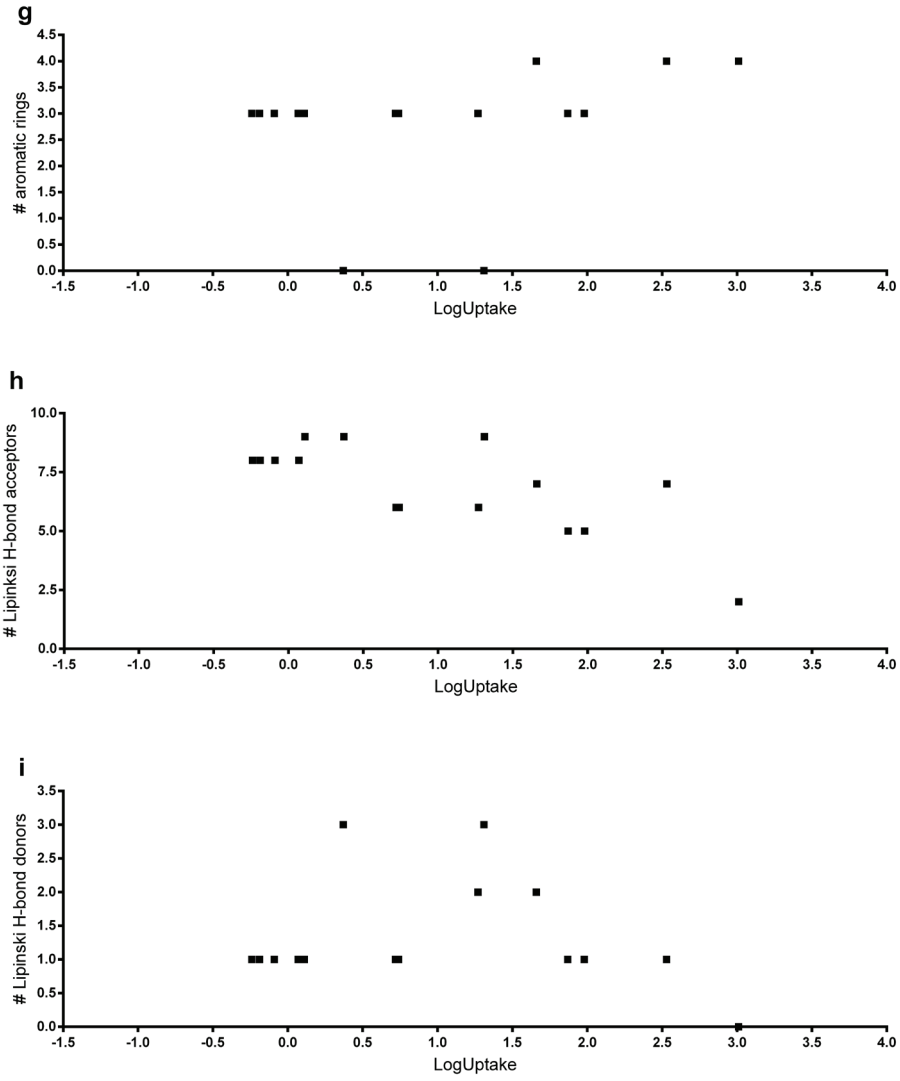

Supporting Figure 2: Uptake versus physico-chemical properties. Common metrics used in predictive strategies was compared to the uptake levels of the 15 compounds used in our assays. In the examination uptake level was compared to (a) molecular weight, (b) molecular volume, (c) number of rotatable bonds, (d) hydrogen bond acceptors and (e) donors, (f) number of sp3 carbons, (g) number of aromatic rings, (h) number of Lipinski H-bond acceptors and (i) donors, and (j) polar surface area.

Supporting Table 1: Minimum inhibitory concentration of compounds

Compound	MIC *M.tuberculosis* (μM)	MIC *M.marinum* (μM)
GSK6	0,08	1,56
GSK10	0,06	0,39
GSK11	1,11	6,25
GSK12	1,59	6,25
GSK13	0,9	6,25
GSK14	0,47	0,39
GSK17	0,03	0,78
GSK19	0,12	0,78
GSK20	0,04	0,39
GSK30	0,06	0,78
GSK32	0,04	0,39
GSK34	0,08	0,78
GSK37	0,22	12,5
GSK38	0,04	0,78
GSK43	1,28	6,25

Supporting Table 2: Log reduction in CFU count in the acute mouse model

Compound	CFU reduction
GSK6	1,65
GSK10	0,1
GSK11	2,85
GSK12	1,76
GSK13	0,03
GSK14	2,92
GSK17	2,98
GSK19	2,52
GSK20	4,65
GSK30	1,95
GSK32	3,7
GSK34	0,99
GSK37	2,3
GSK38	1,14
GSK43	1,06