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High-throughput metabolomic analysis predicts mode of action of uncharacterized antimicrobial compounds

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Abstract

Rapidly spreading antibiotic resistance and the low discovery rate of new antimicrobial compounds demand more effective strategies for early drug discovery. One bottleneck in the drug discovery pipeline is the identification of the modes of action (MoA) of new compounds. Here, we have developed a rapid systematic metabolome profiling strategy to classify the MoAs of bioactive compounds. The method predicted MoA-specific metabolic responses in the nonpathogenic bacterium *Mycobacterium smegmatis* after treatment with 62 reference compounds with known MoAs and different metabolic and non-metabolic targets. We then analyzed a library of 212 new anti-mycobacterial compounds with unknown MoAs from a drug discovery effort by the pharmaceutical company GlaxoSmithKline (GSK). More than 70% of these new compounds induced metabolic responses in *M. smegmatis* indicative of known MoAs, seven of which were experimentally validated. Only 8% (16) of the compounds appeared to target unconventional

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Competing interests: P.P. is an advisor for Biognosys AG and is an inventor on a patent licensed by Biognosys AG that covers the LIP-MS method used in this study: "Method and tools for the determination of conformation and conformational changes of proteins and of derivatives thereof" patent # EP12008011.4. The other authors declare no competing interests. J. L. is an employee of GlaxoSmithKline.

Data and Materials Availability: The compounds used in this study are available from GSK under a Materials Transfer Agreement (MTA). The compendium of drug-metabolome profiles is available in the supplementary tables. Metabolome data can be downloaded from <https://www.ebi.ac.uk/biostudies> with the accession number: S-BSST113.

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cellular processes, illustrating the difficulty in discovering new antibiotics with different MoAs among compounds used as monotherapies. For six of the GSK compounds with potentially new MoAs, the metabolome profiles suggested their ability to interfere with trehalose and lipid metabolism. This was supported by whole genome sequencing of spontaneous drug-resistant mutants of the pathogen *M. tuberculosis* and *in vitro* compound-proteome interaction analysis for one of these compounds. Our compendium of drug-metabolome profiles can be used to rapidly query the MoAs of uncharacterized antimicrobial compounds and should be a useful resource for the drug discovery community.

Introduction

Despite rapid technological progress, the discovery of new antibiotic drugs has been stalled for the past 50 years. To combat the growing burden of antibiotic resistance, innovative drug discovery approaches are required to improve and expedite the antibiotic discovery process (1, 2). To overcome the intrinsic difficulties of target-based screens and to move beyond well-known targets, phenotype-based screening methods are a valid alternative (3). In a classical phenotypic screening approach, promising antimicrobial compounds are selected based on their empirical ability to prevent cell growth *in vitro* (4). However, the lack of efficient, rapid and systematic methods to investigate the modes of action (MoA) of newly discovered cell growth inhibitors has often misguided the selection of the most promising lead compounds (5).

Current systematic inference of MoAs in large chemical libraries is based on large scale *in vitro* direct biochemical techniques (2), comparison of chemical structures (7) or functional genomics approaches. These include *in vitro* resistance mutation mapping (6), cytological profiling (8), monitoring of transcriptional responses (9, 10) and phenotypic assays (11–14) to probe interactions with other drugs (15, 16), gene-deletion/overexpression (17–21) or culture media (22). Although such strategies have been successfully used (22–25), their applicability and scalability is often hampered by cost, reliability and their labor-intensive nature (26, 27).

Emerging applications of metabolomics in drug discovery have focused on biomarker identification and therapeutic monitoring, but only a few are aimed at determining drug MoAs (28–35). Large-scale applications of classical metabolomics techniques are hampered by limited coverage and laborious sample preparation. To address these limitations and to enable systematic testing of large libraries of bioactive compounds *in vivo*, we developed an approach based on non-targeted mass-spectrometry in combination with ad-hoc data mining to enable high-throughput inference of drug MoAs. We first validated our approach on a set of 62 reference compounds with known MoAs applied to the nonpathogenic bacterium *Mycobacterium smegmatis*, and then used this technique to infer MoAs of a library of 212 uncharacterized anti-mycobacterial compounds.

Results

Dynamic metabolome profiling after treatment with reference compounds

We built a reference base of metabolic responses from 62 reference compounds, including currently used antibiotics and chemical stress agents, with 17 known MoAs (Fig. 1A-C; Table S1). These MoAs included inhibition of bacterial DNA replication, protein synthesis, cell wall synthesis and folate synthesis. Reference compounds were administered at three to four concentrations from sub to full inhibition of exponentially growing isogenic *M. smegmatis* in culture at an optical density (OD₅₉₅) of 0.4, in 96 deep-well plates (Table S2; Fig. 1A). The dynamic metabolome response was assessed by withdrawing 80 µl of bacterial culture 5, 30, 60, 180, 360, and 600 minutes after exposure to the compounds. To facilitate dynamic sampling at high throughput and reduce the risk of sample processing artifacts, the whole culture broth was extracted without cell separation using cold solvent extraction and then directly injected into a time of flight mass-spectrometer (36) (Fig. 1A). This procedure enabled rapid profiling of relative dynamic changes of ~15,000 ions, out of which 1,006 could be putatively annotated as deprotonated metabolites. In total, we monitored dynamic metabolite changes across 431 compound-treated conditions and 2 mock treatments [vehicle controls: H₂O or dimethyl sulfoxide – (DMSO)], over 7 time points in three biological replicates (Fig. 1B).

Raw mass spectrometry data were normalized by correcting for instrumental and systematic biases. Before identifying drug-responsive metabolites, we eliminated contaminating ions that were identified in spectra for pure compounds dissolved in water. After removal of drug-associated contaminating ions, a linear model was used to decouple the contribution of the drug treatment to the metabolic changes from plate-to-plate variance, differences in extracted biomass, and background noise. A Z-score normalization was applied before estimating average and standard deviation over the three biological replicates (Table S2 and Fig. S1-S2, see Materials and Methods).

To systematically identify metabolites undergoing significant changes upon treatment with a drug or compound, we used a regression analysis originally described for dynamic transcriptomics data (37). For each compound, dynamic profiles of all metabolites were fitted by a model with five parameters, capable of describing different dynamic adaptive changes of metabolites to new steady state levels. Fit quality estimated by adjusted R² values and the magnitude of metabolite changes were used to select responsive metabolites (Fig. 1D). On average, for each individual perturbation, approximately 5% of the detected metabolites exhibited significant changes (adjusted R² > 0.6 from fitting analysis and absolute log₂ fold-change > 2) (Fig. S3-S4), and the majority exhibited a significant response in at least one of the treatments (Table S3). The onset time of metabolite responses reflected the distance of drug targets to metabolism. For example, very rapid responses occurred for drugs directly targeting metabolic processes, such as inhibitors of folate synthesis, whereas delays in the timing of the response onset occurred upon treatment with antibiotics targeting bacterial protein synthesis or DNA replication (Fig. 1E). Given that a broad common response to bactericidal antibiotics has been suggested (30), we investigated which metabolites exhibited nonspecific responses, common to most perturbations. To this

end, for each metabolite we computed the average of fitting R^2 values across all tested conditions, and the average of largest absolute Z-scores detected along the time course in each treatment. Overall, the commonality of metabolic changes across all tested perturbations was very low (Fig. 2A). Most metabolites affected across multiple conditions were likely associated with general stress responses and exhibited a low but significant correlation with growth inhibition (Fig. 2A and Fig. S5). For example, the accumulation of glycerol 1-phosphate, the first degradation product of glycerol, exhibited a mild but significant correlation ($p \text{ value} \leq 1e-10$) with growth reduction (Fig. 2A). Overall, our data suggested that common changes independent of drug MoAs were an indirect effect of growth-related processes. Given that this conclusion was based on applying most drugs at sub-inhibitory concentrations, future work with these and other bactericidal drugs should examine the presently controversial issue of a common mechanism of antibiotic-induced cell death (38, 39).

Metabolome response similarity and target proximity reflects the drug MoA

Next, we asked whether drug-specific responses occurred in the proximity of corresponding targets and might therefore help to reveal the underlying MoAs. We used a genome-scale metabolic model of *M. smegmatis* (40) to calculate the distance between each enzyme-metabolite pair as the minimum number of reactions connecting the two. To assess whether metabolites exhibiting the largest change in magnitude tended to be located in the proximity of an enzyme, we used a weighted scoring function, in which all metabolic changes were weighted by the respective distance to the tested enzyme. In this analysis, the timing of the response was not taken into account as, for each metabolite, the maximum absolute fold change during the time course was selected. For each treatment and individual enzyme, we systematically assessed the significance of the proximity of metabolic changes by a permutation test (Table S5). Enzymes with a significant enrichment for proximal metabolic changes could be direct drug targets or indirect mediators of the immediate cellular response. Both types of metabolic changes defined what we called the mode of metabolic interference of the compound. To identify the most overrepresented metabolic pathways across compounds with similar MoAs, we calculated the probability for each enzyme to exhibit local metabolic changes within groups of compounds with the same MoA. The resulting top 50 enzymes with consistent low p-values for multiple drugs of the same class, identified across seven main antibiotic categories with known MoAs, were selected and subjected to KEGG pathway enrichment analysis (41) and correction for multiple tests (42) (Fig. 2B, Fig. S6).

Identified genes in most antibiotic classes showed strong functional dependencies for the drug MoA. For instance, enzymes in folate biosynthesis were overrepresented in anti-folate drug treatments, and enzymes related to nucleotide biosynthesis and DNA replication were overrepresented when *M. smegmatis* was treated with quinolones. In contrast, enzymes in amino acid and aminoacyl-tRNA biosynthesis or RNA degradation were enriched upon treatment with protein or RNA synthesis inhibitors, respectively (Fig. 2B). The link between the mode of metabolic interference and the MoA of inhibitors of cell wall synthesis or mycolic acid biosynthesis was less clear, potentially a consequence of the limited lipid coverage by the metabolomics method utilized (Table S6). Nevertheless, the distinct profiles

of metabolites that were often proximal to the target suggested that drugs with common MoAs featured similar metabolic signatures, regardless of whether the target was metabolic or not.

To compare the metabolome responses across multiple drugs, we calculated the similarity of metabolic changes between drug pairs by computing pair-wise mutual information values for each pair of drugs at the corresponding time points. Next, the pair-wise metabolome similarity between time points within 3 to 10 hours after drug exposure were summed up, into one unique similarity score between drug pairs (Fig. S1 and Table S3). The context likelihood of relatedness algorithm (43) was applied to prune false dependencies due to promiscuity in the metabolic responses across different drugs, most likely caused by common indirect growth-related processes (44). By selecting only those perturbations with known targets, we demonstrated that compounds with similar MoAs induced similar metabolic responses (Fig. 3A). On average, compounds targeting non-metabolic processes, such as quinolones and DNA cleavage inducers, or compounds targeting the 30S rather than the 50S ribosomal subunits (Fig. S7), featured more similar metabolic signatures than did antibiotics with similar MoAs but different enzymatic targets (e.g. ampicillin vs cycloserine) (Fig. 3A,B). Overall, we demonstrated that antibiotics with common MoAs had a strong tendency to elicit similar metabolic responses (Fig. S8). We also observed that certain classes of antibiotics, like quinolones for example, exhibited on average higher metabolome similarity than did others, such as cell wall inhibitors (Fig. 3A, B).

To avoid any bias and to predict the MoAs of uncharacterized compounds, we developed a computational framework in which the distribution of pairwise similarities between the uncharacterized compound and compounds with known MoAs was first rank-transformed and Q-values indicating the statistical significance of a MoA as a top ranking antibiotic class were calculated with an iterative hypergeometric distribution formula (45). To test the ability of metabolome-based similarity to predict the MoAs of potential new antimicrobial compounds, we used a procedure in which a reference agent, from MoAs with more than one reference compound, was treated as an uncharacterized compound and its MoA predicted *de novo*. Metabolome-based predictions of MoAs exhibited good agreement with known MoAs [Area Under the ROC Curve (AUC)=0.87; Fig. 3C]. Hence, we concluded that dynamic metabolome changes induced by treating *M. smegmatis* with antimicrobial drugs reflected functional drug properties and could be used to infer the underlying MoAs. Given that different concentrations of the same antibiotic typically elicited similar metabolite responses (Fig. S9), we simplified the screening of new compounds by using a single drug dosage.

Metabolomics-based prediction of MoAs for uncharacterized compounds

Given the potential capacity to distinguish known MoAs through dynamic metabolome responses, we set out to classify MoAs for 212 uncharacterized antimycobacterial compounds with chemical structures that differed from existing antibiotics, which were identified in a large-scale phenotypic screen by GlaxoSmithKline (GSK) (4, 46). These compounds were able to kill *M. tuberculosis* *in vitro* with Minimum Inhibitory Concentrations (MIC) below 10 μ M and with limited toxicity to human cell lines; structures

of these compounds have been made available (<http://dx.doi.org/10.6019/CHEMBL2095176>). *M. smegmatis* was challenged with 10 μ M of each GSK compound. Comparing the measured dynamic metabolome responses to those of the 62 reference compounds and testing the significance of overrepresented antibiotic classes among the most similar reference compounds allowed us to predict MoAs for these GSK compounds. GSK compounds that did not meet a minimum enrichment q-value of 0.05 were not classified in any of the tested MoAs. Most GSK compounds exhibited a significant metabolome similarity to more than one MoA, with the vast majority associating to two MoAs. However, in most cases, the two predicted MoAs were functionally similar, e.g. quinolones and DNA cleavage agents or inhibitors of mycolic acid synthesis or cell wall synthesis (Fig. S10). Although our methodology could not fully resolve the molecular target of an uncharacterized compound solely from the metabolome profile, it did provide a robust prediction of the affected cellular process. For the GSK compounds with at least one significant (q-value $\leq$ 0.05) prediction, we focused on the most significant associated MoA (Fig. 4A, Table S4). Although most GSK compounds were selected to be chemically distinct from known antibiotics (Fig. S11 and Table S2), more than 70% of the tested GSK compounds were classified as having already known MoAs (Table S4). Notably, our data did not enable us to differentiate whether these compounds interfered directly with classical antibiotic targets, whether they bound to different proteins in the same cellular process, or whether they indirectly interfered with transcriptional regulation (47), proteome interactions (48) or enzymatic activation (i.e. pro-drugs) (49).

To validate our predictions for known MoAs, we focused on antifolate drugs that were among the first effective antimycobacterial agents and quinolones that exhibit a large spectrum of activities. Reference folate biosynthesis inhibitors have different targets (50). Trimethoprim, for example, directly targets the dihydrofolate reductase FoaA in a noncompetitive manner, whereas others, like sulfamethizole, are competitive inhibitors of the dihydropteroate synthase FopP. Yet others, like para-aminosalicylic acid (PAS), are pro-drugs with no clear molecular target (51). Because 4 of the 5 predicted anti-folate GSK compounds exhibited high metabolome response similarity to trimethoprim, we tested the inhibitory activity of the 5 compounds on FoaA. With a coupled *in vitro* enzyme assay, we demonstrated direct inhibition of *M. smegmatis* FoaA by 4 GSK compounds (Fig. 4B). The remaining compound, GSK275628A, elicited a metabolome response most similar to PAS (Table S4). In the case of a PAS analogue, enzymatic activation would be necessary to achieve FoaA inhibitory activity (Fig. 4B) (51). Hence, the result from the *in vitro* enzyme assay was consistent with our metabolome-based predictions, suggesting a similar MoA to a prodrug for GSK275628A.

Given that quinolones are able to kill mycobacterial strains resistant to first-line anti-mycobacterial drugs (52), we attempted to validate the predictions for 4 GSK compounds with similar metabolic signatures to quinolones. We tested the induction of DNA damage by each individual GSK compound using a GFP reporter plasmid of RecA promoter activity (53) in exponentially growing *E. coli*. RecA is a key regulatory protein that induces the SOS response upon DNA damage (39, 54). Consistently, with our metabolome-based predictions, we demonstrated that 3 out of the 4 GSK compounds predicted to be quinolone-like agents increased RecA promoter activity (Fig. 4C). As a control, we tested ampicillin, DMSO and 3

randomly picked GSK compounds (GSK2534991A, GSK1826825A, GSK1518999A) predicted to be a protein synthesis inhibitor, folic acid biosynthesis inhibitor and with an unknown MoA, respectively. All negative controls failed to elicit a RecA response, reinforcing the specificity of the assay and the validity of our predictions (Fig. 4C). The quinolone-predicted GSK compound that failed to elicit RecA overexpression (BRL-7940SA) also did not exhibit any inhibitory activity, potentially because it could not penetrate the cell envelope of *E. coli*. Next, we asked whether any of the 4 predicted quinolone compounds could directly inhibit the gyrase complex. We tested direct gyrase binding with an *in vitro* DNA supercoiling assay (Fig. S12). One of the 4 predicted quinolone-like antibiotics, GSK1066288A, directly inhibited activity of *M. tuberculosis* and *E. coli* gyrases, comparable to the 4th generation quinolone antibiotic moxifloxacin (Fig. 4D, E and Fig. S13).

Given that the majority (i.e. 77%) of the 212 tested GSK compounds exhibited responses similar to known MoAs (Fig. 4A), our results suggested that the GSK library contained only a relatively modest number of compounds that could potentially affect non-conventional cellular processes. Among the about 25% of GSK compounds without a metabolome response similarity to known MoAs, 33 did not show significant response differences (q-value = 0.05) to the control conditions. These compounds were either not active under the tested conditions, possibly hinting at a low penetration, or the metabolic response was beyond our detection or coverage limits. The MoAs of these compounds need to be further characterized directly in *M. tuberculosis* (Fig. S14). For the remaining 16 compounds with a metabolic signature different from any of the reference compounds, we identified enzymes in close proximity to the detected metabolic changes and searched for enriched metabolic pathways (Fig. S15 and Table S2). These metabolome-based analyses suggested unique abilities of these compounds to interfere with nitrogen metabolism, oxidative phosphorylation, and the metabolism of fatty acids, vitamin B12 or glyoxylate (Fig. S15). The highly similar metabolome responses for 6 of the 16 compounds suggested a common MoA (Fig. 5A).

To investigate the potentially new MoAs for these 6 GSK compounds, we analyzed their similar responses more deeply. The most common and prominent metabolic changes occurred in α,α -trehalose-6-phosphate and neighboring metabolites in lipid metabolism, carbon metabolism and the cobalamine/heme biosynthesis pathway (Fig. S16 blue dots). GSK1829729A and GSK1829728A are chemically similar to tetrahydropyrazo[1,5-*a*]pyrimidine-3-carboxamide (THPP). Recent pull-down experiments with *M. bovis* demonstrated that THPP binds to EchA6, an enzyme homologue of enoyl-coenzyme A (CoA) hydratase that is involved in fatty acid synthesis (55). Consistently, our network-based analysis of metabolic changes induced by GSK1829729A and GSK1829728A identified local changes in the proximity of several enoyl-CoA hydratase reactions (Table S5). To test whether the six GSK compounds acted through a MoA similar to that of THPP, we selected *M. tuberculosis* H37Rv and Beijing GC1237 mutants that were spontaneously resistant to GSK2623870A.

Spontaneous drug-resistant *M. tuberculosis* mutants have been extensively used to resolve the MoAs of bioactive chemicals, as the mutations can occur directly in the drug target (6),

or may impact biological processes functionally related to the drug MoA (17). Mutants occurred at a frequency of 5×10^{-7} on solid media containing 29.76 $\mu\text{g/ml}$ of GSK2623870A (16x MIC). Despite the difference in chemical structures between THPP and GSK2623870A, whole genome sequencing revealed a single nucleotide polymorphism in the *mmpL3* gene (Rv0206c) of both H37Rv and Beijing GC1237 *M. tuberculosis* mutants (Fig. 5B). Notably, similar resistance mechanisms were observed for THPP (50), N-benzyl-69,79-dihydrospiro[piperidine-4,49-thieno[3,2-c]pyrans (SPIROS) (56) and N-(2-adamantyl)-N-[(2E)-3,7-dimethylocta-2,6-dienyl]ethane-1,2-diamine (SQ109) (57). MmpL3 is an essential inner membrane transporter in *M. tuberculosis*, responsible for translocating mycolic acids in the form of trehalose monomycolates across the cell membrane (58, 59). It is also involved in heme uptake (60), potentially explaining the metabolome changes in heme biosynthesis seen in *M. smegmatis* in response to all six GSK compounds (Fig. S16).

MmpL3 has recently been proposed as a promising target for developing drugs against *M. tuberculosis* (61, 62). MmpL3 has been found to be the target of various antimicrobial drugs with different chemical scaffolds (56, 59, 61), and different mutations in *mmpL3* have been associated with drug resistance (56, 57, 59, 64). Moreover, MmpL3 inhibitors have shown synergistic interactions with first and second line antimycobacterial drugs (57, 63). Despite the apparent attractiveness of MmpL3 as a drug target (65), the vulnerability of this protein to the action of compounds with different scaffolds has generated controversy regarding the importance of the differences in compound chemical structures and their MoAs (55, 65).

To better characterize the MoA of GSK2623870A, we used a chemical proteomics strategy that combined limited proteolysis with mass spectrometry to probe protein conformational changes on a proteome scale (66–68). Protein extracts of *M. tuberculosis* harvested during exponential growth were incubated with the GSK2623870A compound for 5 minutes before conducting limited proteolysis. The analysis identified 30,532 peptides mapping to 2564 unique proteins in *M. tuberculosis* (Table S8). For each peptide, we quantified the difference in abundance between the treated and untreated proteome samples. Differences in peptide abundance reflected protein conformational changes induced by the action of the compound. Such conformational changes can reflect direct binding events or protein network rearrangements by disruption of protein-protein interactions. For 69 peptides mapping to 60 unique proteins, we detected significant changes between the treated and untreated conditions (absolute $\log_2$ fold-change ≥ 2 and pvalue ≤ 0.01) (Fig. 5C). Whereas no significant changes were associated with MmpL3, we found several conformational changes in proteins involved in fatty acid metabolism. In particular, the fatty acid synthase FAS-I protein (Rv2524c) exhibited multiple conformational changes together with many of its interacting proteins, such as FadA, FadA3 and FabD (Fig. 5C and Fig. S17-18). This finding was consistent with the most rapid metabolic changes (absolute $\log_2$ fold-change ≥ 1 and $R^2 \geq 0.6$, 5 minutes after treatment) induced by GSK2623870A. These metabolic changes occurred for several metabolites in close proximity to FAS-I, exemplified by the depletion of the enzyme substrate malonyl coenzyme A (Fig. 5D). Proteins with a conformational change included the known penicillin target PbpA, as well as proteins involved in the tricarboxylic acid cycle. Collective evidence suggests a complex MoA for GSK2623870A due to potentially multiple targets or an indirect role for MmpL3 as a drug importer (55).

Discussion

We describe here a general approach to systematically quantify and interpret dynamic metabolome responses at high-throughput in order to predict the MoAs of small molecules, independent of whether their targets were metabolic or not. The large compendium of drug-metabolome profiles that we generated could be used as a reference dataset to assess similarity in the responses of mycobacteria to new antimicrobial compounds. We predicted and experimentally validated compounds in the GSK library and identified new inhibitors of DNA replication and folate biosynthesis. Network-based analysis of dynamic drug-metabolome changes resolved distinct metabolic responses that were specific to drug MoAs, and were used here to identify compounds with potential new MoAs against mycobacterial targets. Metabolic changes could be detected in the absence of growth inhibitory conditions (Fig. S19), representing a key advantage over classical bacterial growth assays. This feature was crucial to enable identification of a group of six antimycobacterial compounds with similar yet distinguishable metabolic signatures. Whole genome sequencing revealed that MmpL3 mediated resistance to one of the GSK compounds in *M. tuberculosis*, and limited proteolysis analysis suggested fatty acid metabolism was the main cellular process in *M. tuberculosis* targeted by this compound.

Overall, our results suggested that the compound GSK2623870A mainly affected fatty acid metabolism, similar to THPP, expanding the diversity of chemical compounds that potentially could be used to target metabolic processes upstream of mycolic acid biosynthesis. Although the exact functions and roles of MmpL3 in mediating the MoA of this GSK compound still remain to be elucidated, the mutant and proteome results confirmed the initial metabolome-based prediction of an unconventional MoA for this group of compounds. Whereas the 16 anti-mycobacterial compounds with predicted unconventional MoAs exhibited very modest inhibitory activity in *M. smegmatis*, they elicited subtle but unique metabolic patterns reflecting the underlying specificity of their MoAs. Although these compounds are promising candidates for discovering new targets, future studies should investigate the direct metabolic impact of these compounds on *M. tuberculosis* in order to better characterize their MoAs. Nevertheless, the relevance of these compounds tested in *M. smegmatis* for *M. tuberculosis* illustrates that our approach was able to classify compound MoAs in a manner largely independent of the organism. In contrast to traditional chemogenomic methods that are often restricted to model organisms, our metabolome-based approach did not require libraries of mutant bacteria and so can be applied to any bacterial species. For reasons of technical simplicity and cost, it might be preferred to perform the first large scale profiling of compounds in a related, non-pathogenic bacterium given that many MoAs will be similar and drug efficacy can be driven by factors such as drug penetration.

Current state-of-the-art methods to monitor and interpret dynamic drug-induced cellular responses at the genome level do not scale with the size of typical compound libraries used in drug discovery (28–30, 32, 38, 69). Although traditional omics methods such as proteomics, transcriptomics, or genome sequencing (70) provide a large information output, our non-targeted metabolomics workflow enabled rapid measurements at a 10–100 fold higher throughput. Clearly, no single technology provides a general solution for

identification of a compound's MoA or specific molecular target. Our approach, however, could play a crucial role in the initial characterization of new compounds, and could provide complementary data for the interpretation of cytological and phenotypic assays (12, 13). One major limitation of our approach is the difficulty in decoupling metabolic changes that are direct consequences of drug-target interactions, from indirect adaptive metabolic adaptations. At the cost of a lower throughput, time-resolved metabolic profiles with seconds resolution could better resolve direct drug effects and facilitate interpretation of drug-induced metabolome changes. However, it is worth noting that if drug targets are inactive or have low expression under the conditions tested, drug effects will be more subtle or impossible to detect. Mimicking *in vivo* conditions in an *in vitro* setting can improve the efficacy of phenotypic-based assay screens and maximize the output of a metabolomics approach.

Our metabolome-based screening approach can be applied directly to extract multiple quantitative signatures indicative of functional properties of MoAs in large compound libraries. A major advantage of our systematic and rapid methodology for predicting MoAs in large chemical screens is the discrimination of compounds with previously known MoAs from those that have new MoAs. Knowing the MoAs of compounds at an early stage of drug discovery can guide the selection of the most promising leads, even in cases where the drugs do not yet exhibit strong bactericidal or bacteriostatic effects. For drug development, MoA identification is important for follow-up studies, biomarker development, and drug target identification. Even after a relevant drug target is established, additional functional information from our metabolomics drug profiling strategy could help to identify unwanted off-target effects or establish new roles for the target protein within its biological network. Hence, combining our metabolomics and computational based methodology with other omics strategies and more targeted validation technologies should help to overcome the current challenge of predicting the biological effects of drugs before clinical trials begin.

Although the set of reference compounds used in this study was restricted to antibiotics with the most common MoAs, future studies may benefit from a larger set of characterized compounds, regardless of their antimicrobial activities. We envisage that by increasing the number of tested organisms and reference compounds, alternative computational approaches, such as machine learning (71), could open up new opportunities in antibiotic discovery. Beyond inference of the MoAs of bioactive compounds, our approach could be used to identify direct and indirect enzymatic targets of non-lethal compounds, paving the way to predictive models and rational design of effective combinatorial treatments (71, 72).

Materials and Methods

Study design

This study was designed to evaluate the ability of a mass-spectrometry approach to predict the MoA of small molecules by monitoring dynamic metabolic responses of mycobacteria after drug treatment. To this end, we established a new combined experimental-computational framework for large-scale profiling of bacterial metabolic responses to treatment with bioactive compounds. This approach was first tested in *M. smegmatis* on a reference set of 62 compounds applied at different dosages. Rigorous analysis of

metabolomics data demonstrated that antibiotics with a similar MoA elicited similar metabolic changes. This strategy was used to characterize the MoAs of a set of 212 new antimycobacterial compounds, the result of an antibiotic discovery effort of the pharmaceutical company GlaxoSmithKline. This library was used under the terms and conditions of a Material Transfer Agreement (MTA) between GlaxoSmithKline and ETH Institution.

Strains and media

M. smegmatis MC²-155 was used throughout this study. Standard 7H9 minimal medium was used to perform the metabolome drug screening experiment. 1 liter medium contained 2.5 g Na₂HPO₄, 1 g KH₂PO₄, 0.5 g (NH₄)₂SO₄, 0.5 g L-glutamic acid, 0.1 g NaCl, 0.1 g MgSO₄·7H₂O, 40 mg ferric ammonium citrate, 1.8 mg ZnSO₄·7H₂O, 1 mg CuSO₄, 1 mg pyridoxine, 0.5 mg CaCl₂, 0.5 mg biotin, 2 g glycerol as carbon source and 0.05 % v/v tyloxapol. *M. tuberculosis*: H37Rv (ATCC 27294), Beijing GC1237 (74), *M. smegmatis* mc²155 (75), *M. marinum* M (Aranson 1926) and *M. aurum* (76) and their derivatives were cultured at 37°C or 32°C, in the case of *M. marinum*, in Middlebrook 7H9 medium supplemented with ADC (0.5% (w/v) bovine serum albumin, 0.2% (w/v) dextrose, 0.085% (w/v) NaCl, 0.0003% (w/v) beef catalase; Difco), 0.5% (w/v) glycerol and 0.05% (w/v) Tween 80 or on solid Middlebrook 7H11 medium supplemented with OADC (0.05% (w/v) oleic acid, 0.5% (w/v) bovine serum albumin, 0.2% (w/v) dextrose, 0.085% (w/v) NaCl, 0.0003% (w/v) beef catalase; Difco).

Metabolomics drug screening

M. smegmatis was precultivated at 37°C and 300 RPM for 24 hours, diluted 1 to 80 in 125 ml fresh medium in 1 L shake flasks and grown for ~15 h. 700 µL cell cultures were distributed in 96 deep well plates at an OD of approximately 0.4 and incubated for 1 hour at 37°C and 300 RPM before exposure to the drug treatment by the addition of 10 µl drug solution. 62 reference compounds were chosen from well-characterized antibiotics and stress factors, covering most of currently available antibacterial MoAs (see Fig. 1C and Table S1 for more details), together with 212 antituberculosis compounds, obtained from GSK. All stocks were prepared in DMSO solution, except the following ones which were prepared in H₂O due to the low solubility of the compounds in DMSO: CPR, GEN, GET, NEO, H₂O₂, Parq, NaOH, CuCl, EDTA, CTX, OCI and PMB. The GSK compounds were used at a single concentration of 10 µM, while the reference compounds were administered in at least 3 different dosages, aiming for ~100%, ~50% and ~20% inhibition of growth rate (Table S2). All treatments were measured in 3 biological replicates. DMSO and H₂O treatments were included on every plate as control, resulting in 433 different treatments.

Samples were taken immediately before treatment and after 5, 30, 60, 180, 360 and 600 minutes from drug exposure. At each time point, 40 µl of whole cell broth were transferred to 120 µl medium on microtiter plates and optical density was measured at 595 nm. In parallel, 40 µl of the cultures were directly transferred to 120 µl extraction liquid solution containing 50% (v/v) methanol and 50% (v/v) acetonitrile at -20°C. The extraction was carried out by incubating the samples for 1 hour at -20°C. Samples were centrifuged for 5

minutes at 4000 RPM and 80 μ l of the supernatant was transferred to 96 well storage plates and stored at -80°C .

Physiology

Growth rates of *M. smegmatis* upon drug treatment were calculated as the slopes of linear fits to log-transformed OD curves experimentally determined from 30 to 360 min after drug exposure (Fig. S5). In few cases, apparent negative growth rates caused by cell death were set to zero. The inhibition ratios reported in table S4 equal one minus the ratio between the growth rate of the treatment and the average growth rate of the controls on the same plate.

Mass spectrometry measurement, spectral data processing and annotation

The analysis was performed on a platform consisting of an Agilent Series 1100 LC pump coupled to a Gerstel MPS2 autosampler and an Agilent 6550 Series Quadrupole Time of Flight mass spectrometer (Agilent, Santa Clara, CA) as described previously (36). Mass spectra were recorded from m/z 50 to 1000 using the highest resolving power (4 GHz HiRes).

All steps of mass spectrometry data processing and analysis were performed with Matlab (The Mathworks, Natick). For each acquired spectra, ion-peaks were identified with the *findpeaks* function of the Signal toolbox. Identified peaks with less than 5000 ion counts were excluded from further analysis. Detected ions were matched to a list of metabolites based on the corresponding molar mass. Since there is no available manually curated genome-scale model of *M. smegmatis* metabolism, we compiled a comprehensive list of metabolites from the following models: *M. tuberculosis* H37Rv model from the Model SEED database (Seed83332.1 (77), http://blog.theseed.org/model_seed) and the manually curated *M. tuberculosis* model from (78); *E. coli* K12 model iJO1366 (79); *M. smegmatis* MC²-155 model from the Biocompare database (BMID000000141548 (80), <https://www.ebi.ac.uk/biomodels-main>). For the full list of metabolites used for annotation see Table S6. The chemical formula of each metabolite was used to calculate the deprotonated monoisotopic molecular mass. Detected ions within a mass tolerance difference of less than 0.003 Da were associated to the nearest reference metabolites. In total 1006 different reference masses could be detected (see Table S2-S6 for the reference masses and the associated metabolites).

Supercoiling assay

To test gyrase activity, the *M. tuberculosis* and *E. coli* DNA gyrase assay kit from Inspiralis and TopoGEN, respectively, were used. The procedure adopted to monitor DNA gyrase dependent supercoiling followed kit instructions. Reaction was performed in 30 μ l volume by incubating at 37°C for 45 minutes approximately 250 ng of pHOT1 Relaxed DNA together with the purified gyrase protein. Moxifloxacin or the compound GSK1066288A was added to the reaction mixtures at different concentrations. Termination, detection, and readout were carried out as described in the kit protocol using agarose gel electrophoresis. Bands were quantified using imageJ. Raw gel pictures can be found in the Supplementary.

Folate assay

The capability of compounds to inhibit the folate biosynthesis was tested with an enzyme assay. Full protein extract was prepared from *M. smegmatis* the following way. 70 ml cell culture in exponential phase (at about OD of 1) was harvested and centrifuged for 10 min at 4 °C and 5000 RPM. The precipitate was resuspended in 8 ml lysis buffer (100 mM Tris buffer (pH = 7.5), 4 mM PMSF, 2 mM DTT, 5 mM MgCl₂). French press was used to extract the cells. The samples were centrifuged for 10 min at 4 °C and 5000 RPM and the supernatant was filtered to concentrate the protein extract to approximately 2 ml (centrifuged at 4 °C and 5000 RPM in an ultrafiltration tube). The reactions were performed in 150 µl on a microtiter plate. The ratio of the (buffer: protein extract) was 1:2 in the solution. The reaction was started by adding 0.1 mM and 0.4 mM of dihydrofolate and NADPH, respectively. A plate reader was used to measure the decrease in the NADPH level at 340 nm for 1 h, sampling the absorbance every 26 sec.

Selection of spontaneous GSK2623870A resistant mutants of MTB H37Rv and Beijing GC1237 and whole-genome sequencing (WGS)

GSK2623870A spontaneous resistant mutants were selected on 7H11 plates supplemented with OADC and 16X MIC of antibiotic at 37°C after 3 weeks of incubation. Genomic DNA was isolated from saturated cultures of the different mycobacteria and resistant isolates. Sequencing was carried out by GATC (Constance, Germany), using Illumina Myseq 300 and HiSeq4000 instruments. Identification of SNPs, insertions and deletions was done using different bioinformatics tools as GATK modules, PICARD and snpEff. Raw sequence reads were deposited in the NCBI sequence read archive (SRA) database under the accession number SRP116576.

Statistical analyses

Statistical analyses were performed using Matlab R2015b (The Mathworks, Natick). In total, we monitored dynamic metabolic changes across 431 compound treatment conditions and 2 mock treatments (vehicle control H₂O or dimethyl sulfoxide - DMSO), over 7 time points in three biological replicates. To assess differences in metabolite abundance before and after treatment, we used a multiple linear regression scheme. Similarity of metabolic profiles between different treatments was assessed using an entropy-based measure of similarity (Mutual Information), and prediction of compound MoAs was based on an iterative hypergeometric test described in (45). When necessary, p values were corrected for multiple tests by q-value estimation. Metabolomics data and analysis results are available as supplementary tables.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Single Sentence Summary

The mode of action of new antimicrobial compounds can be predicted from changes in metabolome profiles in *Mycobacterium*.

Editor's Summary

Boosting the Antibiotics Pipeline

Antibiotic discovery requires innovative phenotypic assays to identify the mode of action of compounds during largescale drug screening. Now, Zampieri et al. have developed a high-throughput method to systematically quantify and interpret dynamic metabolome responses of mycobacteria to new antimicrobial compounds. They demonstrate how one can infer the mode of action of uncharacterized anti-mycobacterial compounds by comparing drug-induced metabolic responses in *Mycobacterium smegmatis*. Their combined mass-spectrometry-based metabolomics and computational workflow scaled with the size of typical compound libraries and hence could facilitate the selection of antimicrobial compound leads with potentially new modes of action.

Figure 1. Antibiotic-induced metabolome responses in *M. smegmatis*.

(A) Metabolomics workflow. Cells were grown in 700 μ l volumes in 96 well plates to an OD₅₉₅ of approximately 0.4, before addition of 10 μ l of the antimicrobial compound. 80 μ l of cell culture was withdrawn from each well at each sampling time. 40 μ l was used to determine cell density, and the remaining 40 μ l was added to cold extraction buffer. Supernatant was directly injected into a time of flight (TOF) mass spectrometer and relative changes in metabolite intensities were extrapolated from processing of the metabolome data.

(B) Compounds tested. Almost half of the compounds tested included different

concentrations of reference antimicrobials (yellow) and chemical stress agents (green) with known MoAs; the remainder were compounds from a GSK library used at 10 μ M concentration (blue). (C) Distribution of MoAs for the 62 reference compounds. (D) Schematic representation of the drug-metabolome response data set. For each antimicrobial compound tested, the dynamic profile of 1006 metabolites was interrogated. As an example, the top graph illustrates the response of the folic acid biosynthesis intermediate 4-aminobenzoic acid to the antimicrobial para-aminosalicylic acid (104 μ M, red; 41 μ M, grey, 25 μ M, blue). The bottom graph shows the response of the bacterial metabolite mycobactin to the known antimicrobial isoniazid (1.5 mM, red; 0.22 mM, grey; 0.11 mM, blue). Thick lines represent the results from the impulse model fitting analysis for the three drug concentrations. Metabolic profiles of 4-aminobenzoic acid and mycobactin across all conditions are shown in light grey. (E) Distribution of metabolic response onset times for antibiotics belonging to the seven main antibiotic categories tested in this study. The onset time is defined as the time at which metabolite changes reached half of their maximum change after treatment of *M. smegmatis* with the compounds. For each perturbation (treatment with compound), metabolites with a model fitting $R^2 \geq 0.6$ and a maximum absolute $\log_2$ fold-change ≥ 2 were retained.

Figure 2. Commonalities among metabolite changes in response to antimicrobial treatment.

(A) Correlation between growth rate and metabolite abundance in *M. smegmatis* after treatment with antimicrobial compounds. Each dot represents a metabolite. The two axes represent the mean R^2 across all tested conditions and the mean of maximum Z-scores across all tested conditions and time points. Color reflects the degree of Spearman correlation between maximum Z-score and growth inhibition across all tested conditions. Metabolites with an average R^2 0.5 and $\log_2$ Z-score 0.5 are shown. (B) Pathway enrichment for metabolome responses to antibiotics with seven known MoAs: (1) - cell wall synthesis inhibitors; (2) - DNA cleavage, (3) folic acid biosynthesis inhibitors, (4) quinolones (5) mycolic acid biosynthesis inhibitors, (6) protein synthesis inhibitors, (7) RNA synthesis inhibitors. Enrichment was performed with the 50 most frequently identified genes for each antibiotic class. The heatmap shows enriched KEGG metabolic pathways with q-values 0.01.

Figure 3. Pair-wise similarity of antimicrobials with respect to metabolic changes induced in *M. smegmatis*.

(A) Similarity heatmap for 62 reference antimicrobials. Similarity calculated between each drug-perturbed condition is represented as a symmetric heatmap. Diagonal values are not taken into account and are set to not-a-number (grey). Highlighted boxes correspond to the seven main MoAs of the 62 reference compounds. (B) Magnification of panel in A showing antimicrobial compounds that blocked gyrase and cell wall synthesis in *M. smegmatis*. (C) Receiver Operating Characteristic (ROC) curve measuring the ability of metabolome-based predictions using the iterative hypergeometric test (45) to discriminate antibiotics sharing

similar MoAs. Notably, we considered only MoAs that applied to more than one antimicrobial reference compound. CPR, ciprofloxacin; LVX, levofloxacin; MFL, – moxifloxacin; NAL, nalidixic acid; NFL, norfloxacin; OFL, ofloxacin; AMX, amoxicillin; AMP, ampicillin; CCL, cefaclor; CTX, ceftriaxone; OXI, oxacillin; BAC, bacitracin; CYC, D-cycloserine; EMB, ethambutol; FOS, fosfomycin.

Figure 4. Metabolome-based predictions of MoAs for 212 GSK compounds.

(A) Grouping of metabolome similarity-based predictions for the 212 GSK compounds into known MoAs. (B) Impact of antimicrobial drugs on normalized Folate *in vitro* activity. The measured dihydrofolate conversion rate was normalized to the activity measured with DMSO vehicle only. All tested compounds were dissolved in DMSO: 40 μM of streptomycin (STR), 40 μM and 1333 μM of trimethoprim (TRM, TRM-H), 40 μM para-aminosalicylic acid (PAS) and 40 μM of six GSK compounds. (C) Shown is RecA promoter activity in exponentially growing *E. coli* treated with the following: four GSK compounds predicted to be quinolone-like agents (BRL-79405A, BRL-109885A, GSK1066288A, GSK695914A), norfloxacin, ampicillin, DMSO and three GSK compounds (GSK2534991A, GSK1826825A, GSK1518999A) predicted to be a protein synthesis inhibitor, a folic acid biosynthesis inhibitor or with an unknown MoA, respectively. (D) Gyrase activity of *M. tuberculosis* measured using an *in vitro* supercoiling assay at different concentrations of moxifloxacin (MFL) or GSK1066288A. (E) Gyrase activity of *E. coli* in the presence of DMSO, MFL, STR or GSK1066288A. Activity of denatured gyrase was used as a negative control (∅).

Figure 5. Analysis of metabolite changes in *M. smegmatis* after treatment with the GSK compound GSK2623870A.

(A) Pairwise similarity between *M. smegmatis* metabolome response profiles to 16 GSK compounds with no similarity to known MoAs. (B) Schematic representation of trehalose monomycolate exporter protein MmpL3. Transmembrane segments are represented in violet. Circles indicate the locations of amino acid changes associated with resistance of *M. tuberculosis* to antimycobacterial lead compounds previously found to select for resistance mutations in MmpL3. These compounds include SQ109 *N*-(2-adamantyl)-*N*-[(2E)-3,7-dimethylocta-2,6-dienyl]ethane-1,2-diamine (blue), THPP tetrahydropyrazolo[1,5-a]pyrimidine-3-carboxamide (orange) and SPIROS *N*-benzyl-69,79-dihydrospiro[piperidine-4,49-thieno[3,2-c]pyran] (purple). The black star indicates the amino acid change associated with resistance to the GSK compound GSK2623870A. The genomes of the *M. tuberculosis* H37Rv and Beijing GC1237 mutant strains contain an A to G single nucleotide polymorphism at position 755, which resulted in a tyrosine to cysteine missense mutation at position 252 of the MmpL3 protein. (C) Results from limited proteolysis analysis. Each dot in the volcano plot represents the relative difference in peptide abundance

between the treated and untreated proteome extracts. Proteins highlighted in red are known to physically interact with fatty acid synthase FAS-I (Fig. S17, S18). For each protein, the size of the dot reflects the number of interacting partners (73) with significant conformational changes. (D) Shown are rapid metabolic changes induced by the GSK antimicrobial compound GSK2623870A. Each dot corresponds to the R^2 and Z-score values of the metabolite 5 minutes after exposure of *M. smegmatis* to the antimicrobial compound. Metabolites highlighted in red are involved in fatty acid metabolism.