TITLE

Emerging approaches to tuberculosis drug development: At home in the metabolome

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ABSTRACT

Once considered a crowning achievement of modern drug development, tuberculosis (TB) chemotherapy has proven increasingly unable to keep pace with the spread of the pandemic and rise of drug resistance. Efforts to revive the TB drug development pipeline have, in the meantime, faltered. Closer analysis reveals key experimental deficiencies that have hindered our ability to ‘reverse engineer’ knowledge of antibiotic mechanism into rational drug development. Here, we discuss the emerging potential of metabolomics, the systems level study of small molecule metabolites, to help overcome these gaps and serve as a unique biochemical bridge between the phenotypic properties of chemical compounds and biological targets.

The Rising Tide of Antimicrobial Drug Resistance

Few therapeutic agents rival the societal impact of antibiotics. Following their introduction into clinical medicine, antibiotics transformed once fatal infections into predictably curable maladies, revolutionized the fields of surgery, maternal-fetal health, oncology, and transplantation medicine, and launched parallel revolutions in the scientific fields of microbiology, biochemistry and genetics. Unfortunately, the impact of this once historic legacy has since been eroded by the steady rise of resistance.

Resistance is a natural consequence of antibiotic use whose emergence was first predicted with the introduction of antibiotics into clinical practice [1]. The failure of anti-infectives research to keep pace, however, was not foreseen. A key contributing factor to this shortfall is the experimental dichotomy between antibiotic activity and mechanism. Though experimentally simple to assay, most antibiotics work through a complex series of events refractory to any single experimental readout. As a result, early antibiotic development flourished under the empirical simplicity of phenotypic screening, while efforts to ‘reverse engineer’ a mechanism-based pipeline languished in experimentally fragmented form

The advent of biologically unbiased systems level (-omic) technologies has recently begun to remedy this situation. Metabolomics is the youngest of these systems-level disciplines and focuses on the global complement of small molecules (or metabolites) in a biological system under a given set of conditions [2]. From a biological perspective, metabolites constitute the final products of enzymes and enzyme networks which catalyze the biochemical activities of all cells [3]. Growing evidence has further shown that metabolites serve cellular physiology in equally specific qualitative and quantitative ways [4]. Metabolomics has thus emerged as a systems-level lens into the biochemical state of a cell or organism, similar to what nucleic acid- and peptide-based readouts achieved for genes/transcripts and proteins [3]. From an analytical perspective, metabolomics offers the unique ability to directly monitor the intracellular levels and fates of a given compound within a bacterial cell. In the context of drug development, the ability to elucidate both the intrabacterial ‘pharmacokinetic’ fates and ‘pharmacodynamic’

actions of a given compound within the native biochemical milieu of a cell has poised metabolomics to uniquely address key gaps in anti-infectives research.

Here, we review emerging roles of metabolomics in early tuberculosis (TB) drug development. Though often considered a disease of antiquity, TB remains the leading infectious cause of deaths worldwide [5]. Lacking a vaccine capable of conferring predictably consistent protection in adults [6], control of the pandemic has come to rest on the combined efficacy of infection control and chemotherapy [5]. Unfortunately, treatment for even drug sensitive disease remains longer and more complex than for virtually any other bacterial infection, fostering unintended rates of treatment default and paradoxical emergence of drug resistance itself [7,8].

Historically speaking, nearly all clinically approved TB drugs emerged from empirical approaches that have since proven unproductive [9]. The need for new TB drugs has thus prompted concerted efforts to develop more rational approaches [10]. Below, we discuss specific roles of metabolomics in target discovery methods and their potential to improve the precision and efficiency of rational drug development.

Target-Based Drug Development

Target-based drug development is a target-first, ‘forward’ chemical biology paradigm that emerged from the molecular revolution [11]. Based on the ability to study and manipulate individual genes and proteins, it became possible, if not attractive, to identify therapeutically relevant targets for use as biological anchors in drug development. Target-based approaches thus introduced the conceptually aesthetic notion that potency, selectivity and toxicity were chemically discrete properties that could be rationally engineered into drugs in a modular fashion. Due to their simpler requirement for *in vitro*, rather than *in vivo*, target inhibition, target-based approaches also enabled broader and more sensitive screening of chemical compounds than traditional phenotypic approaches [12,13].

Target-based drug development begins with the discovery of a specific gene or protein whose dysregulation elicits the therapeutic effect of interest. This process is then followed by high-throughput screens (HTS) to identify potent and selective *in vitro* chemical mimetics that can be developed into compounds with *in vivo* activity in experimental models of increasing complexity and, ultimately, effectiveness in people, as exemplified in the area of infectives by the development of HIV-1 integrase inhibitors [14].

From a historical perspective, target-based programs took root in *in vitro* biochemical assays of target activity that were limited in biological scope, but were quickly supplanted by genome-scale profiling and mutagenesis technologies that enabled organism-wide surveys for potential targets with gene-specific precision. The impact of genome-based technologies, however, was tempered by the sizeable (~40%) annotation gap of most microbial genomes and limited accuracy of homology-based methods in predicting the biochemical function and/or specificities of a given gene and related paralogs [15]. Target identification and validation have thus emerged as a critical, but often incompletely defined, foundation of all target-based approaches.

Below, we highlight recent advances in metabolomics that have begun to help strengthen and reinforce this critical foundation.

Known knowns: Enzyme (target) annotation

One of the earliest, and most intuitive, applications of metabolomics was the biochemical annotation of metabolic enzymes. By comparing the metabolic profiles of wild type and genetically manipulated cells, metabolomics made it possible to define the *in situ*, rather than *in vitro*, activity of metabolic enzymes within an intact cell based on the accumulation and depletion of corresponding substrate product pairs (Figure 1A) [16]. This application not only helped overcome a key barrier in functional genomics (associated with the limited accuracy and completeness of homology-based predictions), but also enabled the study of enzymes within their native physiologic environments.

In the context of drug development, such capabilities have proven especially valuable. Among clinically approved agents, enzymes constitute the single largest class of known drug targets, a fact that reflects both the essentiality of their biological activities and the structural suitability of their active sites to chemical inhibition [17]. Knowledge of the native biological function of drug targets has proven similarly invaluable in helping to predict late stage failures and, in cases of targets with host paralogs, compound toxicities. The advent of metabolomics thus enabled the specific, but unbiased, identification and characterization of metabolic enzymes as drug targets.

For TB, the publication of the complete genome of *Mycobacterium tuberculosis* (*Mtb*) and genome-wide mutagenesis studies identified hundreds of genes required for growth and survival, launching a new era in TB drug discovery [18–20]. However, surprisingly few pharmacologically tractable targets have emerged to date. This is because, as is the case for most microbes, a large proportion of *Mtb*'s essential genes are annotated only at the level of functional class, while others have been found to be misannotated, others unannotated altogether [18]. The emergence of proteomic techniques, such as activity-based proteomic profiling (ABPP), has begun to fill these gaps but thus far been restricted to class-level functions, such as adenosine nucleotide binding [21] or serine hydrolase [22], due to the limited range and specificity of existing probes.

Limitations notwithstanding, metabolic enzymes have emerged among the most significant mediators of *Mtb*'s pathogenicity. Accordingly, comparative metabolomics has been used to successfully annotate the biochemical and physiologic functions of numerous essential metabolic enzymes [23–26]. For example, Berney *et al.* used data from bioinformatics databases and genetic screens to identify genes essential for methionine biosynthesis in *Mtb*, and identified a presumptive homoserine transacetylase (MetA). MetA catalyzes the first committed step in the biosynthesis methionine, an essential *Mtb* metabolite [23]. To confirm its function, comparative metabolomics profiling of wild type and *metA*-deficient *Mtb* revealed accumulation of several amino acids upstream of MetA, and depletion of metabolites downstream of MetA like homocysteine, methionine and S-adenosylmethionine, demonstrating MetA's function as a homoserine transacetylase *in vivo*.

In a more challenging example, Ehebauer *et al.* used untargeted metabolomic profiling to study the function of the AccD1-AccA1 protein complex that evaded functional annotation by other methods [27]. Unexpectedly, they found that AccD1-AccA1-deficient bacteria accumulated 3-methylcrotonyl-CoA, linking the protein complex to amino acid catabolism [27].

Though seemingly trivial, validation of the *in vivo* function of enzymes is important because even if a protein function is considered known, and biochemical assays confirm the annotated function *in vitro*, its essential *in vivo* function can be different (Figure 1B).

Isocitrate lyase (ICL), for example, is a metabolic enzyme required for *Mtb* to infect and persist in mice [28,29]. This ability has long been attributed to its canonical role in metabolizing even-chain fatty acids through the glyoxylate shunt. Unexpectedly, however, ¹³C stable isotope tracing studies revealed that its essentiality for survival was due to activity as a methylisocitrate lyase, an enzyme of the methylcitrate cycle, whether metabolizing even or odd chain fatty acids [30]. These results thus not only resolved a key ambiguity pertaining to the metabolic essentiality of ICL but also revealed biologically linked potential alternative targets in the methylcitrate cycle.

Mtb's lipoamide dehydrogenase (Lpd) was similarly predicted, by Venugopal *et al.*, to be a part of two known enzyme complexes: pyruvate dehydrogenase (PDH) and peroxynitrite

reductase/peroxidase (PNR/P). However, an *Mtb* strain lacking both PDH and PNR/P activities exhibited a less severely attenuated phenotype than an Lpd-deficient strain, indicating the existence of additional essential functions of Lpd. Accordingly, comparative metabolomics profiling of wild type and Lpd-deficient *Mtb* revealed accumulation of intracellular pyruvate, leucine, isoleucine, valine, and their keto acid metabolites in bacteria, revealing a deficiency in branched-chain keto acid dehydrogenase (BCKADH) activity, which was confirmed by showing that Lpd is an essential component of BCKADH [31].

Building on the theme of unannotated enzymatic multifunctionality, Maksymiuk *et al.* found that deficiency of 2-hydroxy-3-oxoadipate synthase (HOAS) did not result in general metabolic perturbations corresponding to a previously identified *in vitro* role as the E1 component of *Mtb*'s putative α -ketoglutarate dehydrogenase complex, suggesting an alternative *in vivo* function [32]. Comparative metabolomics profiling and additional biochemical work showed instead that HOAS was part of a four-component peroxidase system that protects *Mtb* against glutamate anaplerosis and nitroxidative stress [33].

Together, these examples highlight the broad biochemical power of metabolomics to enable the unbiased discovery and annotation of the physiologic function of essential metabolic drug targets.

Known unknowns: Pathway annotation

Enzymes rarely function in isolation. As a result, their essentiality is often linked to, if not defined by, the pathway or multiple pathways they serve. Understanding the pathway(s) which define the biochemical function of a given target can thus not only serve to elucidate the biological basis for its therapeutic relevance (and potential efficacy) but also reveal additional targets capable of synergizing with, or even replacing, the original target (Figure 1C).

Genetic studies of *Mtb* virulence, for example, first identified the *pckA*-encoded phosphoenolpyruvate carboxykinase as an enzyme required for *Mtb* to establish and maintain infection in mice [34,35]. PckA was annotated to catalyze the reversible interconversion of

oxaloacetate (OAA) and phosphoenolpyruvate (PEP). However, further studies revealed that *pckA* was specifically required for growth on fatty acids, a physiologic carbon source thought to be encountered by *Mtb* *in vivo*, while ^{13}C tracing studies specifically demonstrated that this essentiality was linked to the predominantly unidirectional conversion of OAA to PEP [34]. These results thus implicated gluconeogenesis as a key physiologic determinant of *pckA*'s essentiality. This importance was confirmed, amongst others, by Ganapathy *et al.*, who showed the essentiality of fructose biphosphatase (FBPase), a dedicated gluconeogenic enzyme that catalyzes the unidirectional conversion of fructose 1,6-bisphosphate to fructose 6-phosphate [36,37]. Although *Mtb* was annotated to encode a single FBPase (*glpX*), a combined genetic and metabolomics approach revealed a second FBPase activity (*gpm2*) capable of supporting gluconeogenesis in the absence of *glpX*, leaving GlpX a poor drug target [36]. Nonetheless, knockout of both *glpX* and *gpm2* proved lethal, confirming the essentiality of the gluconeogenesis pathway as a whole [36].

In a more physiologic approach, two independent studies identified an essential role for succinate metabolism in *Mtb*'s ability to adapt to hypoxia, a physiologic feature of many of its host niches. Upon exposure of *Mtb* to hypoxia, Watanabe *et al.* found a considerable increase in extracellular succinate, which they linked to reverse tricarboxylic acid (TCA) cycle activity by isotopic tracing of ^{13}C -glucose [38]. They also reasoned that *Mtb* could maintain an energized membrane by operating the reductive branch of the TCA cycle and secreting succinate. Knockout of the canonical fumarate reductase (*frd*), however, did not reduce succinate secretion [38]. Work by Eoh and Rhee used ^{13}C -glucose and ^{13}C -acetate isotopic tracing to monitor *Mtb*'s metabolic adaptations while switching from normoxia to hypoxia and back [39]. These studies showed that, upon adapting to hypoxia, *Mtb* slowed TCA cycle activity and increased succinate production through ICL, which had previously been thought to act only in fatty acid metabolism. Moreover, by fermenting fumarate to succinate, *Mtb* was shown to be capable of sustaining both membrane potential and ATP synthesis at varying oxygen levels [39]. These two studies thus identified enzymes involved in succinate metabolism- such as malate dehydrogenase and succinate dehydrogenase- as potential drug targets against non- or slowly replicating *Mtb*.

From a broader perspective, these examples exemplify the potential for metabolomics to serve as an activity-based guide to biologically relevant target proteins and pathways.

Unknown unknowns: de novo annotation

Though powerful in genomic scope, a cardinal limitation of most nucleic acid-based approaches is the sizeable (~40%) annotation gap of nearly all sequenced microbial genomes, including that of *Mtb* [18]. Less well recognized, however, is the fact that up to 30% of detected enzymatic activities remain genomically unannotated [40]. Notwithstanding the experimental challenge of annotating these gaps, such genes and activities comprise a potentially valuable, but untapped, source of essential and, owing to their lack of recognizable sequence homology, species-specific metabolic drug targets. Accordingly, recent work has begun to leverage the biologically agnostic analytical power of untargeted metabolomics to mine this pool [41]. Similar to known unknowns, *de novo* annotation of unknown unknowns can be accomplished by phenotype-first or protein-first metabolomics approaches.

In an early ‘forward’ approach, De Carvalho *et al.* devised a protein-first *in vitro* assay called activity-based metabolite profiling (ABMP) to detect and identify the function of unannotated ‘orphan’ metabolic enzymes. In this assay, a purified recombinant form of the protein of interest is incubated with a mycobacterial small molecule extract and analyzed for reaction products that exhibit a time- and protein-dependence through untargeted metabolomics comparisons (Figure 1E). Applying ABMP to the essential gene product of Rv1248c, de Carvalho *et al.* observed a matching time- and protein-dependent consumption of α -ketoglutarate and production of 5-hydroxylevulinate. Subsequent work confirmed the activity of Rv1248c, which was annotated as a thiamine-dependent α -ketoglutarate decarboxylase, as a 2-hydroxy-3-oxoadipate synthase, whose primary product underwent spontaneous decarboxylation to 5-hydroxylevulinate [42]. Importantly, the ability to observe this reaction in an *Mtb* strain overexpressing Rv1248c enabled physiologic validation of an otherwise genetically essential reaction.

ABMP was similarly applied to annotate Rv1692, which had been annotated as a nucleotide phosphorylase, but was unexpectedly found to function as a D, L-glycerol 3-phosphate phosphatase. This finding, in turn, led to the discovery of glycerophospholipid recycling – a pathway that was not known to exist in *Mtb* [43]. The ability to pre-select for proteins without structural homology to known proteins and intrinsic selectivity for metabolic activity, thus make ABMP uniquely suited to find novel metabolic targets that can be selectively inhibited.

Using the model organism *E. coli*, Sévin *et al.* recently increased the scalability of ABMP further by enabling its use with either purified protein or enriched cell extracts followed by high throughput (infusional) mass spectrometry using an analytically standardized format [44].

In a functionally orthogonal approach, [45]Layre *et al.* conducted an untargeted metabolomic comparison of *Mtb* with that of non-virulent *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) to identify *Mtb*-specific virulence lipids (Figure 1D) [46]. This elegant work identified a highly abundant molecule, 1-tuberculosinyladenosine (1-TbAd), which was found on both the cell surface and in the shed extracellular media. A targeted metabolomic analysis of a transposon library of *Mtb* mutants for 1-TbAd then identified Rv3378 as its corresponding biosynthetic enzyme, deletion of which both abrogated production of 1-TbAd and attenuated its virulence. A subsequent untargeted comparison of the culture supernatant of nine *Mtb* strains versus nine non-tuberculous *Mycobacterium* strains confirmed the specificity of 1-TbAd, but also identified three 1-TbAd derivatives, dexpanthenol, Val-His-Glu-His and PG(16 : 0/0 : 0) and several unknowns as additional *Mtb*-specific metabolites [47]. These studies thus not only identified *Mtb*-specific biomarkers, but also revealed the corresponding enzymes and potentially novel *Mtb*-specific drug targets.

In a conceptually similar vein, Meissner-Roloff *et al.* conducted an untargeted metabolomic comparison of a hypo- and hyper-virulent Beijing strain of *Mtb*, and found a plethora of metabolic differences, though the biologic bases of these differences, whether genetically or physiologically mediated and their functional significance, were left unresolved [45].

COMPOUND-BASED DRUG DEVELOPMENT

In contrast to target-based drug development, compound-based drug development is a compound-first, 'reverse' chemical biology paradigm. As such, compounds are selected on the basis of the desired phenotypic effect, regardless of the underlying mechanism or target. This approach offers the advantage of simultaneously pre-selecting for activity and cell penetration, two key barriers associated with target-based programs, while requiring no knowledge of a specific target or mechanism-of-action (MOA). As a result, compound-based screens have gained preference over target-based screens in the recent years [48,49].

In practice, the lack of such knowledge poses a significant barrier to compound-based approaches due to difficulties in avoiding nuisance compounds (such as non-specific cytotoxic agents like detergents), recognizing compounds that work through biologically irrelevant MOA's [50], and guiding structure-activity relationship (SAR)-based optimization of compound potency and safety, whose application is especially powerful when combined with structural biology [51].

A key limitation of compound-based approaches is thus the limited precision of existing MOA identification tools. Traditional biochemical methods for example, focus on primary compound-target interaction and use of binding affinity as a surrogate of target identification that, while specific, is not always functional or sufficiently sensitive to yield a specific target. In contrast, transcriptome-based approaches, while biologically broader in scope, report on the response, rather than direct impact, of a given compound, and thus make it hard to identify targets beyond the pathway level, or in comparison to a compendium of previously characterized reference compounds [52]. Whole genome sequencing of drug-resistant clones, though uniquely powerful for their organism-wide scope, is similarly associated with complex isolation procedures and the not infrequent discovery of secondary resistance genes involved in drug-activation, drug efflux and associated DNA transcription, while missing the primary target [53–55]. Existing technologies have thus left key gaps in the compound-based pipeline.

Mechanism-of-Action Discovery

Identification of metabolic blocks

Inhibition of a metabolic enzyme generally results in increased levels of its substrates and decreased levels of its products. Metabolomic profiling has thus been applied to identify and characterize the MOA of compounds acting through metabolic targets (Figure 2A).

In a recent study of inhibitors of the D-Ala pathway to peptidoglycan synthesis, Prosser *et al.* noted that the canonical alanine racemase (Alr) inhibitor β -Chloro-D-Ala (BCDA) exhibited poor *in vitro* inhibition of *Mtb* Alr but potent inhibition of *Mtb* growth [56]. Isotopic tracing of ^2H - and ^{13}C -labeled alanine surprisingly revealed substantial D-Ala-D-Ala formation in the presence of BCDA, indicating that neither Alr nor the related enzyme D-Ala-D-Ala ligase (Ddl), was the target of BCDA. Metabolomic profiling of BCDA-treated *Mtb* instead revealed a decrease in D-Glu incorporation into muropeptides, that was accompanied by matched increased levels of N-glycolated muramic acid (MurNGly) and L-Ala pools, and a depletion of L-Ala-D-Glu dipeptide pools. *In vitro* experiments subsequently confirmed that BCDA was an inhibitor of glutamate racemase (Murl), another essential enzyme early in peptidoglycan synthesis [56].

In a combined genetic-metabolomic approach to identify the *in vivo* target of D-cycloserine, Halouska *et al.* compared the metabolomic profile of wild type *Mycobacterium smegmatis* to those of *M. smegmatis* strains that lacked or overexpressed alanine racemase (Alr), following treatment with D-cycloserine [57]. Although the untreated wild type and Alr knockout strains had different metabolite profiles, D-cycloserine treatment exhibited similar metabolic effects on all strains. The lack of a metabolic block specific to wild type cells thus suggested that Alr might not be an important target of D-cycloserine. In subsequent work, both Prosser *et al.* and Halouska *et al.* showed that D-cycloserine potently inhibited the next enzyme of the pathway, D-Ala-D-Ala ligase (Ddl) and that growth inhibition by D-cycloserine treatment coincided with a block in D-Ala-D-Ala synthesis in both *M. smegmatis* and *Mtb* [58,59].

Together, these studies highlight the ability of metabolomic profiling to both identify the target of a metabolic inhibitor and also distinguish between closely related compounds working on different targets in the same pathway.

Mechanism-of-Action Classification

In addition to serving as a specific lens into metabolic drug targets, metabolomics has also enabled biologically unbiased classification of compound MOAs in a way similar to what was achieved with genome-wide RNA profiling [52,60–62]. This application makes specific use of the biological discriminatory power, rather than biological information, of metabolomic profiles as a means of classifying experimental compounds in relation to a compendium of reference profiles of known MOA (Figure 2B) [63].

Using an NMR-based platform, Halouska *et al.* were able to successfully classify three hit compounds from a whole cell screen against *Mtb* as exhibiting similarity to inhibitors of cell wall biosynthesis among a reference panel of 12 drugs of known MOA. These same reference profiles, however, also failed to distinguish inhibitors of transcription, translation and DNA supercoiling [64]. The degree of biological resolution achievable with these profiles thus remains to be determined.

Intrabacterial Pharmacokinetics

Building on the analytical, rather than biological, power of metabolomics, recent work has made it possible to directly track the intracellular ‘pharmacokinetic’ (PK) levels and fates of compounds within bacteria (Figure 2C). Irrespective of target or MOA, compound-based drug development operationally focuses on optimizing a specific *in vivo* binding interaction, the identity of which is the goal of MOA studies. A significant, but often unrecognized, barrier to this approach is a general lack of knowledge concerning the identity of the *in vivo* ligand. Despite its pre-selection for both phenotypic activity and cell penetration, compound-based approaches often fail to distinguish whether a phenotypically active compound is the biochemically active species or a pro-drug and, if a pro-drug, what the identity of the relevant metabolite is.

In the context of TB drug development, it may be of particular interest to note that many of the TB drugs in current clinical use and development are pro-drugs that undergo extensive biotransformation in *Mtb* [9,65]. Within this context, metabolomics have helped to identify N-methylation as a mechanism of drug resistance [55].

In a contrasting study of the clinically active drug para-aminosalicylic acid (PAS) and the phenotypically inactive sulfonamide, sulfamethoxazole, Chakraborty *et al.* showed that, while both compounds exhibited *in vitro* activity as competitive inhibitors of *Mtb*'s dihydropteroate synthase, PAS functioned as a replacement substrate that underwent biotransformation into dysfunctional folate analogs; in contrast, the more potent but phenotypically inactive sulfamethoxazole underwent extensive steric modification [66]. These studies thus revealed the unique analytical potential and value of metabolomics in elucidating the intrabacterial PK of compounds in TB drug development.

Metabolomics, in addition, offers the ability to simultaneously monitor the PK of a compound and its biological effect on bacteria. Such an integrated approach that offers a simultaneous readout for both the pharmacokinetics and pharmacodynamics properties of a compound would be particularly useful for drugs that are metabolically inactivated [55], or activated [53,67]. The thiophenecarboxamide derivative 7947882, for example, requires activation by the monooxygenase EthA to inhibit the CTP synthetase PyrG, which then leads to decreased CTP levels and characteristic downstream effects on the metabolome [67].

Taken together, these examples highlight the unique ability and value of metabolomics to simultaneously elucidate both the intrabacterial pharmacokinetic fates and pharmacodynamic actions of a given compound within the native biochemical milieu of a cell.

Drug Resistance

Mtb is intrinsically resistant to most antibiotics [68]. As described above, part of this resistance is due to its impermeable cell envelope, metabolizing enzymes and efflux transporters, but part also lies in *Mtb*'s metabolic plasticity and the ability to enter into metabolic quiescence.

Nandakumar *et al.* used metabolomics to search for common metabolic changes in response to isoniazid, rifampicin and streptomycin treatment [69]. Although these antibiotics act against different targets, they unexpectedly triggered similar TCA cycle remodeling that indicated activation of ICL activity. Antibiotic-induced ICL activation resulted in a net change in the reductive arm TCA cycle, which was found to protect *Mtb* against oxidative stress. Interestingly, ICL-deficient *Mtb* was found to be hypersensitive to the antibiotics, which could be rescued with antioxidants. These results not only show that ICL's are involved in antioxidant response, they also show that all three antibiotics induce oxidative stress.

Intrinsic resistance aside, acquired resistance to antibiotics is mediated by heritable genetic mutations. In many cases, these mutations result in specific changes that pertain to alterations in access to or the activity of the cognate biochemical target. However, recent work has begun to suggest that these same mutations may also be associated with specific secondary metabolic alterations in the basal physiology of drug resistant organisms.

For example, mutations in the beta subunit of RNA polymerase (*rpoB*) render *Mtb* insensitive to rifampicin. Using GC-MS on a small number of *rpoB* mutants, du Preez and Loots found decreased levels of 10-methyl branched-chain fatty acids and cell wall lipids, suggestive evidence of increased metabolism of short fatty acids [70,71]. Using LC-MS lipidomics, Lahiri *et al.* reported altered levels of mycobactin siderophores and acylated sulfoglycolipids [72]. Mutations in catalase-peroxidase (*katG*) that render resistance to the frontline drug isoniazid, by preventing activation into its biochemically active form, were similarly associated with increased levels of several fatty acids, alcohols and alkanes [73].

Unfortunately, while provocative, such studies remain descriptive and in need of independently controlled validation and functional significance. Nonetheless, the potential for biomarkers of drug resistance or anti-resistance targets themselves remains intriguing.

Concluding Remarks

Looking ahead, it is exciting to consider emerging and future roles of metabolomics technologies in drug development. In addition to the specific challenges discussed here, numerous others remain. For example, in characterizing or pre-validating potential drug targets, it remains an important, but unaddressed, need to determine their vulnerability to chemical inhibition. Drug targets, by definition, must be amenable to binding of a chemical compound to a degree that is sufficient to elicit the biological phenotype of interest. Some enzymes, even when essential, are present in such biological excess that the level of inhibition required to stop cell growth is too high for the target to be druggable. Conversely, other enzymes that have no biological margin for reduced activity are likely to be highly vulnerable to inhibition and thus pharmacologically tractable, if not, attractive targets. Genetic approaches to rank targets based on altered levels of protein abundance have recently confirmed that known TB targets can exhibit a significant range of vulnerabilities [74–79]. However, protein levels are not always a perfect surrogate of enzyme activity. Metabolomic profiling of target activity, in combination with genetic and chemical inhibitors, thus offers a potentially valuable means of determining target vulnerability in a pharmacologically unified manner, as was demonstrated for dihydrofolate folate reductase and its inhibitor trimethoprim [76]. Moreover, such an approach could also help assess how far a lead compound is from achieving clinically relevant inhibition.

Target discovery and validation aside, a key barrier to both target- and compound-based approaches is the inability to resolve target access from target engagement and target inhibition. Classical SAR-based drug development focuses on the SAR between the compound and its cognate target. However, the phenotypic activity of a compound arises from the contributions of numerous additional biological factors that include the ability of a compound to cross the bacterial envelope and accumulate within the target cell, its proclivity to undergo or avoid activating or inactivating modifications, and its ability to ultimately reach its target; each of which is either currently unmeasured or monitored in a fragmented manner. The ability of metabolomic profiling to directly and simultaneously monitor the biochemical fates and impact of compounds within *Mtb* may make it possible to elucidate and help optimize the

activity of chemical compounds along each of these distinct pharmacokinetic and pharmacodynamic SAR axes (see outstanding questions).

In the same way, it is interesting to consider whether these same approaches might also enable compounds to be developed along yet another set of SAR axes that pertain to selectivity and host toxicity.

A key operational barrier to realizing the potential impact of metabolomics however, remains the still nascent nature of its technological face. Nonetheless, given the increasing number of metabolomics core facilities and standardization of analytical practices, we expect that metabolomics will become an increasingly accessible resource to investigators both within and outside of anti-infectives research [80].

Future notwithstanding, the applications highlighted in this review establish a unique and promising role for metabolomic approaches in modern TB drug development.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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FIGURE LEGENDS

Figure 1. Metabolomic applications in target-based drug development. (A) Metabolomic profiling of mutant strains can point to the function of potential drug targets by revealing specific metabolic blocks and; (B) reveal unexpected multifunctionality of enzymes that were expected to catalyze only a single reaction, while (C) metabolomic profiling of mycobacteria exposed to physiological stresses can reveal pathways that are essential to survive these stresses, as well as specific targets and potential mechanisms of resistance. (D) Comparative metabolomic profiling of virulent versus non-virulent strains can reveal virulence-associated metabolites whose biosynthetic pathways represent drug targets. (E) Activity-based metabolite profiling is an *in vitro* technique to identify the function of enzymes of unknown function. Colored dots represent metabolites.

Figure 2. Metabolomic applications in compound-based drug development. (A) Metabolomic profiling of drug-treated bacteria can reveal specific metabolic blocks that reveal the mode of action of a compound. (B) Metabolic responses to drugs cluster based on mode-of-action, allowing classification of compounds with unknown mode-of-action. (C) Metabolomics can be used to simultaneously monitor the pharmacokinetic properties of a drug and its metabolites, and their pharmacodynamic effects on the metabolome. D: Drug; M: Metabolite. Colored dots represent metabolites.

Figure