Metabolomics and Lipidomics applications in the context of immune and cancer cells metabolism

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Chapter 2

Recent advances of metabolomics analysis in early drug development

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Abstract

The pharmaceutical industry has early on adapted proteomics and other omics' technologies for drug research. Although metabolomics lacked behind in this development it has nowadays become an accepted and widely applied approach in early drug development. Over the past decades metabolomics evolved from a pure exploratory tool to a much more mature and quantitative biochemical technology. Today, several types of metabolomics based platforms are applied during the early phases of drug discovery. Metabolomics analysis assists in the definition of physiological response and target engagement markers as well as the elucidation of the mode of action of drug candidates under investigation. In this brief review we will highlight recent examples and novel developments of metabolomics analysis applied in early drug development.

Keywords

Metabolomics, Drug Discovery, Lipidomics, NMR, LC-MS, Flux, Target

Teaser

Metabolomics has become an integral part of drug discovery and development. Its importance for the pharmaceutical industry will keep growing as will its manifold applications in today's drug discovery process.

Introduction

The drug development process can roughly be divided into five stages: early drug discovery, preclinical studies, clinical development, regulatory review, and post-market monitoring [1,2] **(Figure 1)**. During the early stages potential drug candidates are evaluated to confirm and validate their target engagement (TE) and mode of action (MoA). Particularly these stages involve the intensive use of bioanalytical methodologies and strategies. Next to traditional technologies, as for example, enzymatic assays, electrophoresis and spectroscopy; mass spectrometry (MS) has taken center-stage as highly versatile and valuable technology crucial to many aspects of modern drug development [3]. In addition to more established applications, such as metabolite monitoring and identification [4,5], or its application as highly sensitive detection technology for pharmacokinetics or pharmacodynamics studies [6-8], MS has been adapted by the pharmaceutical industry early on particularly in proteomics applications [9]. However, metabolomics, the comprehensive study of an organisms' biochemical composition, also having roots in the pharmaceutical industry, was much later tailored for drug discovery and drug development programs [10]. Nevertheless, as outlined by Riley and Tymiak and discussed here, metabolomics applications have great potential for accelerating drug development at several stages [11]. Key application areas of metabolomics during the drug development process include: i) target identification, ii) MoA elucidation, iii) discovery of TE markers, as well as iv) physiological response (PR) markers including therapy monitoring. In turn, the use of MS and nuclear magnetic resonance spectroscopy (NMR) based metabolomics has become a fundamental technology across the early stages of drug development. An excellent overview about the application of metabolomics in unravelling (patho-) physiological mechanisms has just recently been given by David Wishart [12]. In this review, we provide a brief introduction to metabolomics, and we present an update of the existing literature about metabolomics technologies applied to substrate and target identification, elucidation of the MoA, definition of TE and PR markers, as well as, possible future applications as for example drug repurposing.

Metabolomics technologies: a brief overview

Several in-depth reviews are covering the field of metabolomics analysis [12-15] and we here discuss the most recent developments and give a brief overview in the context of drug discovery. Generally speaking, all metabolomics driven projects should start with a detailed study design and clearly defined research objectives and goals. In other words, metabolomics analysis is not a biochemical silver bullet but has to be applied in a dedicated and focused way if meaningful results are to be obtained.

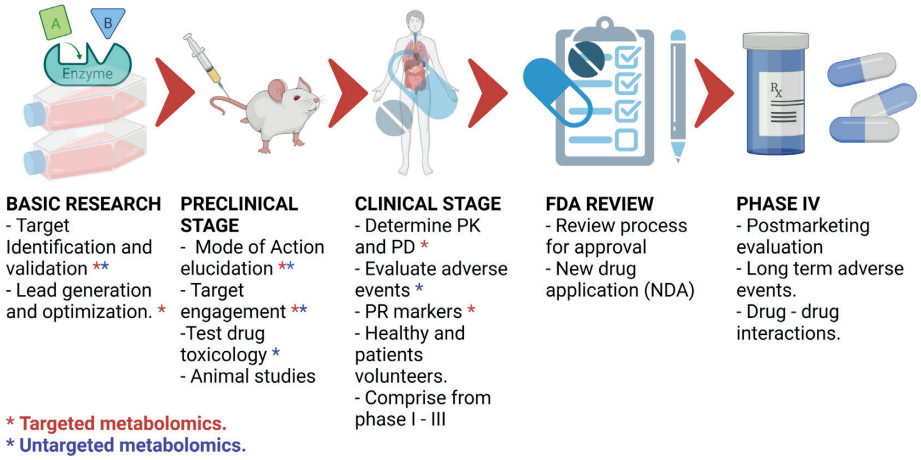

Figure 1: The drug development process and the application of metabolomics analysis. The drug development process spans from basic research to post marketing evaluation of a drug. Each of these steps comprises different goals aimed to describe the pharmacodynamics and pharmacokinetics as well as the toxicology and side effects of investigational new drugs. Due to its high sensitivity and specificity, metabolomics has become an integral part of this process.

Based on the envisaged research goals suitable biospecimen have to be collected and correctly stored until analysis[16]. Subsequently, and in light of the envisaged outcome the most suited chemical analysis platform(s), being either a targeted or untargeted (see below) approach is being chosen. After successful chemical analysis, data quality has to be assessed. This is frequently done by comparing pooled study samples and/or quality controls which should be part of every metabolomics study[17,18]. Thereafter, data visualization and interpretation follow. For visualization, heatmaps as well as volcano plot analysis have become fairly popular. A good starting point and online resource with a significant user base is Metaboanalyst 5.0[18]. Following visualization, data interpretation will be guided by the project aims. However, translation of metabolomics derived findings into mechanistic insights will always demand a multi-disciplinary approach and experimental, phenotypic validation. Common strategies amongst many others are: pharmacological approaches, siRNA, validation cohorts (biomarkers), or isotope tracing.

Metabolomics concepts

Metabolomics aims at comprehensively mapping all biochemical reactions in a given (biological) system. The approach can roughly be divided into targeted and untargeted approaches [13]. Targeted approaches require prior knowledge of the molecule(s) of interest in order to design tailored analytical methods. However, the establishment

of extensive targeted methods has become increasingly facilitated by the availability of metabolite libraries (e.g. IROA technologies) [19]. Untargeted approaches aim at detecting as many metabolites as possible in a sample with subsequent annotation and identification, usually involving database searches (e.g. METLIN, MS-DIAL)[20,21] as well as comparison with authentic synthetic materials [22]. Targeted approaches have the advantage of facile metabolite identification, whereas untargeted metabolomics analysis includes the additional step of identification [13] and the often unmet requirement of pure chemical standards for verification and quantification [22]. However, there is significant progress in overcoming the challenges of untargeted metabolomics with the generation of large scale mass spectrometric databases (e.g. METLIN, MS-DIAL) tailored to substance identification from untargeted metabolomics approaches [23,24] as well as, integrated software solutions [25,26]. Furthermore, metabolic network analysis [26], molecular formula oriented approaches (HERMES) [27] or in the case of lipids, decision tree approaches [28] have recently been introduced. Moreover, cognitive computing and other advanced computational tools aimed at facilitating metabolite annotation have successfully entered the field [29,30]. An outline of metabolite identification throughout the centuries has just recently been provided by Giera et al. [31].

Analytical technologies

Today, liquid- and gas- chromatography (LC and GC) are the leading technologies used for metabolite separation [32]; whereas NMR and MS dominate the field as detection technologies (**Figure 2**). Lately, gas-phase based separations have also entered the field, e.g. differential mobility spectrometry (DMS) which is used for lipid class separations [33,34] or drift tube-based separations as additional parameter for the unambiguous identification of metabolites [35]. NMR is routinely applied as stand-alone technology not involving prior analyte separation, whereas MS is frequently coupled with LC or GC separations. Both approaches, NMR and MS-based metabolomics come with distinct advantages and disadvantages [36]. MS-based approaches offer particularly high sensitivity, [37,38] with the drawback that analyte identification and quantification demand the availability of genuine standards and ideally isotopically labelled materials [39-41]. Targeted methods come with a risk of being agnostic to any potentially critical metabolite not included in the coupled compound library. Despite this, these methods provide a comprehensive analysis of several hundred to more than one thousand molecular species within a single analysis, making them an excellent first choice for investigating drug induced metabolic alterations and possible enzyme substrates. Another challenge lies in the analysis of isomers and diastereomers which are intrinsically difficult to separate with conventional methods [42]. Diastereomers can

generally be separated using routine methods as for example reversed phase LC or GC separations. Enantiomers on the other hand require sophisticated methods such as chiral derivatization or separation [43]. However, as can be exemplified with D/L-2-hydroxygluratae, enantiomeric resolution can be of clinical relevance. The oncometabolite D-2-hydroxyglutarate is favorably produced over its L-enantiomer when a mutation in the IDH gene triggers a reverse reaction in the TCA cycle [44], the separation of both enantiomers is routinely carried out with chiral derivatization. Nevertheless, this example illustrates one of the future challenges in metabolomics analysis. Compared with MS, NMR suffers from a significantly lower sensitivity (2-3 orders of magnitude) but allows for absolute quantification and facilitates de novo structure elucidation [39-41]. Due to its capacity providing absolute concentrations, NMR has become an established technology for the analysis of abundant intracellular metabolites for example partaking in central energy metabolism, e.g. glucose, lactate, ATP, TCA cycle intermediates and amino acids, as well as, monitoring extracellular exchange of nutrients [45]. Table 1 below summarizes metabolomics approaches and technologies and gives practical examples which are gaining traction in the pharmaceutical industry.

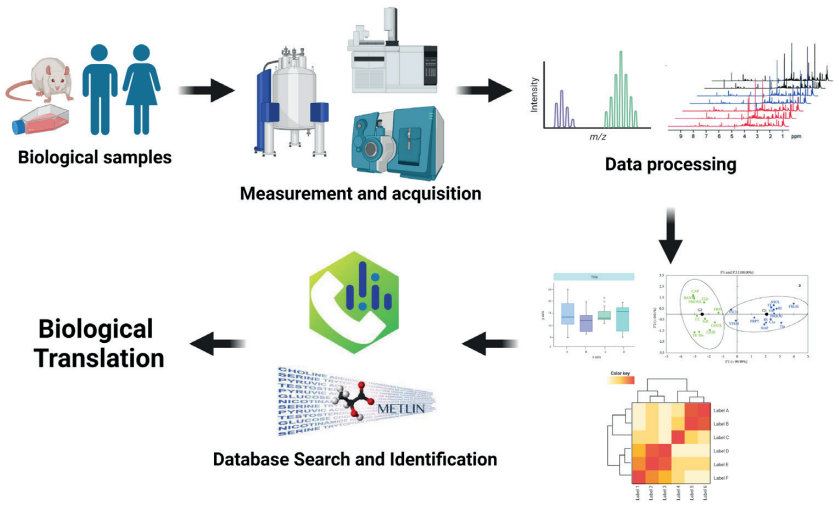

Figure 2: General metabolomics workflow. Following sample treatment analysis is carried out employing a diverse set of analytical technologies, subsequently dedicated data processing and analysis approaches are applied to extract and select the most important changes observed. Finally biological interpretation employing database searches and advanced algorithms such as for example ingenuity profiling analysis are being applied.

New developments and future directions

An extension to "classic" metabolomics-driven experiments, coined as stable isotope tracing, utilizes stable isotope tracers (e.g., ^{2}H, ^{13}C and ^{15}N) incorporated to nutrients such as glucose and glutamine, and provides a dynamic representation of metabolism. Tracing experiments are often coupled to simulated metabolic networks to enable metabolic flux analysis (MFA) [46]. Depending on the investigated pathways and the expected concentrations of the involved metabolites the most suited chemical analysis platform(s) for MFA or isotope tracing is being chosen. NMR-based methods benefit from the quantification of abundant metabolites while simultaneously facilitating the positional analysis of ^{13}C or ^{15}N tracers, even for metabolites with identical molecular weight [47,48]. On the other hand, dedicated MS based platforms have been developed for comprehensive isotopic tracing analysis [49,50]. Just recently a new method coined as comprehensive isotopic targeted - MS (CIT-MS) has been applied for MFA in mammalian cells [51]. A promising approach that builds on the strengths of NMR and MS combining both technologies for quantitative metabolomics [51] and stable isotopic tracing [52]. In addition, both NMR and MS are increasingly being used for stable isotope tracing studies *in vivo* [50], as recently reviewed by Fernández-García et al. [53]. Jointly, these developments bring metabolomics approaches closer to clinical applications and the drug development process. Regardless of the analytical platform, the capability to unravel dynamic interactions of metabolic pathways has led MFA to become increasingly adopted in the field as a key next step towards comprehensive characterization and understanding of metabolism [49]. To this end, an increasing number of studies has started to adopt isotopic tracing and MFA for the analysis of drug induced alterations in metabolism [54-56], particularly in the cancer field, in which isotope tracing has become an established technology [50,57]. Next to the aforementioned metabolomics centered developments is an increasing amount of reports focusing on multi-omics integration [58]. The main aim of integrating two or more 'omics' data sets lies in generating a more comprehensive and solid view of metabolism and disease relevant alterations [59,60]. Recent examples stem from antibiotics [61] and cancer research [61]. Moreover, single cell metabolomics in a multi-omics setting is also gaining traction within drug discovery [62]. Other important recent developments include increased metabolite coverage and structural analysis [63] as well as the use of isotopically labelled cell extracts as internal standard materials for comprehensive metabolite quantification [64].

Table 1: Examples of industrially applied metabolomics approaches.

Approach	Advantage	Drawback	Examples	Future Directions (examples)
Untargeted LC-MS/MS metabolomics	Discovery of unknowns Broadest possible coverage	Hardly quantitative Metabolite identification remains a bottleneck	XCMS [65], MS-DIAL [21], TOXcms [66]	Chiral Metabolomics [67] Advanced informatics for metabolite identification [31,68]
Targeted LC-MS based metabolomics	Known metabolite identities (Semi-) quantitative	Agnostic to unknowns and restricted to the panel.	Biocrates kit [69] Metabolon platform (semi-targeted) IROA metabolite libraries [19]	Chiral Metabolomics [70] Isotopically labeled extracts for quantitation [64]. MFA [53,71]
Flow-injection based platforms	No separation column (Semi-)quantitative	Matrix effects Analyte overlap	Lipidyzer platform [72]	Increasing coverage and structural details [34,63]
NMR based metabolomics	Non-destructive quantitative analysis	Lower sensitivity compared to LC-MS	Fragment-based drug discovery (FBDD) [73,74]	MFA [53]
Mass spectrometry Imaging (MSI)	Direct on-tissue analysis for spatially resolved metabolomics	Complex and time consuming. Fresh frozen materials have to be available.	Matrix-assisted laser desorption/ ionization (MALDI) - MSI [75,76]	On tissue derivatization [77] Multi-omics integration [78]

Metabolomics in the early stages of drug development

Traditionally the first step in any drug development process involved the selection of promising targets for disease modification, followed by extensive screening procedures for possible interactors. However, nowadays novel candidate targets are frequently identified based on 'omics' data such as genomics or transcriptomics [79], linking specific genetic alterations with disease phenotypes [80]. For example, Luukonen et al. just recently showed that carriers of the hydroxysteroid 17-β dehydrogenase 13 (HSD17B13) [81] gene variant (rs72613567:TA) have a reduced risk of non-alcoholic steatohepatitis and cirrhosis [80]. Such targets, identified by population wide screens and associations, frequently enter the drug development pipeline without prior knowledge of their biochemical function. However, detailed biochemical knowledge is of crucial importance to MoA and TE marker identification. In turn, metabolomics approaches have taken center stage for obtaining a detailed understanding of underlying biochemical processes and enabling MoA and TE identification. Ultimately, these steps are highly important for moving promising candidates further in the development process. Additionally, phenotypic drug discovery (PDD), a target-agnostic process, is becoming more widely adapted in the pharmaceutical

industry [82]. In contrast to traditional procedures like target based drug development (TDD), where screening is largely carried out based on a known target, PDD is agnostic to the actual target, thus rendering target identification, MoA and TE marker assessment key supplements for its ultimate success. Altogether, these processes aim to identify and optimize new targets and chemical entities (NCE) before their progression to clinical trials as validated targets and/or investigational new drugs (IND). The outputs from this process help to validate the drug target and to determine the pharmacological properties such as absorption, distribution, and metabolism of the druggable compound [83,84]. In summary, the advent of PDD and genetically derived targets has led metabolomics approaches to take center stage during the early phases of drug development. Key questions which can be addressed by metabolomics approaches, include: i) the definition of endogenous protein (enzyme) substrates, ii) MoA investigations, iii) the definition of TE markers and iv) the prediction of adverse effects (**Figure 3**) [85]. Additionally, metabolomics approaches might also directly pinpoint towards bioactive metabolites, involved in the MoA of an NCE, a research field, recently coined as activity metabolomics [14]. Examples include inhibitors of the enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) aimed at increasing and leveraging levels of endogenous prostaglandin E2 [86,87]. The enzyme dehydrocholesterol reductase 24 (DHCR24) and its endogenous substrate desmosterol [88]. Inhibition of DHCR24 and consequently increased levels of desmosterol have just recently enabled selective liver X receptor activation without undesired co-activation of the sterol response element binding proteins [89]. Another example is indole-3-propionic acid as unusual antibiotic with anti-inflammatory and anti-oxidative properties [90,91]. Interestingly, the concept has already impacted early drug discovery [92] and triggered a new research initiative coined as metabolic medicine [93].

Substrate identification

Hits deduced from PDD or the analysis of genetic mutations can result in candidate targets without sound knowledge of their biochemical properties as for example endogenous precursors and products of a specific enzyme. This lack of detailed biochemical knowledge can severely complicate MoA elucidation as well as, the analysis of (*in vivo*) TE. In this setting, metabolomics approaches are ideally suited to elucidate endogenous enzyme substrates. A typical experiment to achieve this would involve the comparison of the metabolic signatures of siRNA or knock-out *in vitro* and *in vivo* models compared against wild-type controls. Alternatively, "*ex vivo*" metabolomics where a recombinant enzyme is exposed to its near-native metabolic environment [95], the analysis of protein metabolite interactions [96] or proteomics assisted metabolomics involving protein pull down strategies [97] are feasible strategies for substrate identification. An overview of these techniques has

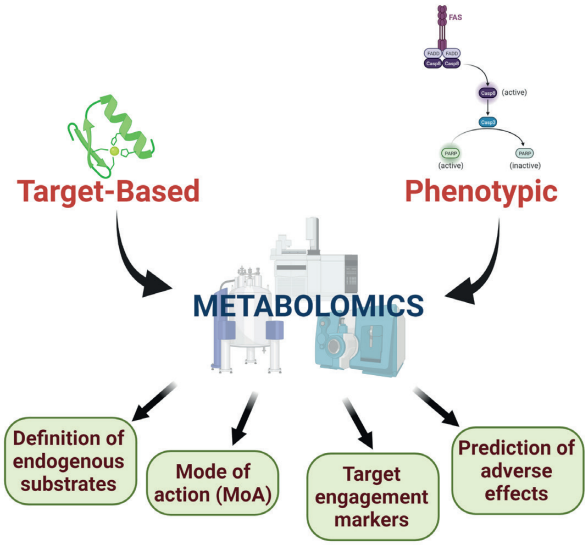

Figure 3: The role of metabolomics in early drug development. Screening of drug candidates can be performed by two different approaches: Target-based drug discovery (TDD) and phenotypic drug discovery (PDD). TDD is a guided process base on "binders", a specific gene product becomes the target of new chemical entities. On the other hand, PDD is a mechanism agnostic drug discovery approach focusing on therapeutics that change a disease phenotype 93. In both TDD and PDD, metabolomics has a central role in the establishment of endogenous substrates, elucidation of the MoA, TE, and/or the prediction of adverse effects.

been presented by Prosser et al. [98]. As analytical read out strategies, comprehensive targeted mass spectrometry based platforms covering hundreds to thousands of metabolites (Table 1), NMR based approaches [99,100], as well as untargeted metabolomics approaches are being applied as referred previously in section 2. The choice of the employed analytical platform depends heavily on the exact research question and *a priori* knowledge of the target under investigation. Regardless the method used, subsequent to chemical analysis the obtained metabolic data from different conditions are being compared and disturbances in metabolic conversions are mapped to establish target lists of candidate substrates for subsequent validation. The interpretation of the data is facilitated by advanced pathway based technologies [101] as for example ingenuity pathway analysis [102]. Although the aforementioned approaches can result in potential enzymatic substrates, their ultimate success is jeopardized by several important factors. Biological systems can pose significant tissue heterogeneity complicating or even, not reproducing [103] the translation of *in vitro* findings in subsequent *in vivo* experiments. Proteoforms [104] might mask (selective) enzyme inhibition as they can take over the enzymatic action of the actual target. A prominent example are phospholipases an enzyme class known to

consist of several proteoforms [105]. Additionally, and in-line with the aforementioned example, metabolism is a dynamic process with many intertwined and reversible pathways. For example, the anaplerosis of the TCA cycle in the mitochondria, might occur from various pathways, including the metabolism of glucose and glutamine. Thus, while the levels of TCA cycle intermediates may remain unaltered by reflecting a metabolic steady state, the actual fluxes and the contribution of substrates might differ. A prominent example is the high demand of glutamine by specific cancers [106]. In order to address specific enzymatic activities, isotopic tracing or flux analysis has been employed [107]. Using this approach in lung squamous cell carcinoma (SCC) it was possible to specifically target glutaminolysis and effectively overcome therapy resistance *in vivo* [108]. Furthermore, the identification of glutamine metabolism as a therapeutic target, was recently explored *in vivo* with a prodrug of the glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON), which is activated by enzymes enriched in the tumor microenvironment to simultaneously shut down glycolysis and oxidative phosphorylation in mouse cancer cells while enhancing T cell oxidative phosphorylation and anticancer immune responses [109]. In another example, isotopic tracing analysis highlighted the importance of one carbon metabolism [110], and especially the role of serine and glycine metabolism in tumor growth [111]. Based on these findings, serine metabolism is now a promising pathway for drug development in cancer therapy, e.g., targeting the enzyme serine hydroxymethyl-transferase (SHMT) which catalyzes the production of glycine from serine [112] or by promoting the formation of 1-deoxyshingolipids via serine restriction by a controlled diet [113]. Other recent examples of identified substrates in cancer pharmacology are presented by Liang et al. [114]. Despite the fact that most of the applications of stable isotope tracing and MFA for substrate identification are from the cancer research field, the applicability of the method extends to other research areas as well. An example is the discovery of trimethylamine-*N*-oxide (TMAO) function as an atherotoxin originating from liver trimethylamine and the gut derived products choline, betaine and carnitine [115]. This finding was achieved by metabolomics in combination with stable isotope tracing and led to the identification of two substrates targeting TMAO levels for the treatment of artherosclerosis, flavin monooxygenase 3 (in the liver) and bacterial choline TMA-lyase [116,117].

Target identification, physiological response markers and mode of action

The most straight-forward application of metabolomics in drug discovery lies in the identification of PR markers. The definition of useful PR markers does not necessarily involve a direct correlation with the MoA of a given target or drug

candidate but rather tries to establish any measurable and quantifiable PR marker induced by a certain target or drug candidate. To this end, PR marker analysis usually proceeds along the lines of "classic" biomarker discovery studies [118,119]. Typical pre-clinical approaches compare knock-out or knock-down strategies with wild types in vitro an in vivo [120]. Moreover, PR marker analysis is of significance for clinical trials and pharmacokinetic and pharmacodynamic modelling [121] and forms the basis of pharmacometabolomics and personalized therapy [120,122]. Unlike the definition of PR markers does metabolomics assisted target identification and elucidation of MoA usually rely on prior knowledge of affected metabolic pathways. In other words, drug-induced metabolic disturbances are analyzed (statistically) using pathway maps and the most significantly disturbed nodes (proteins) are selected for further investigation/validation. Such analysis can be carried out by combining untargeted metabolomics analysis and *in silico* or chemoinformatic approaches [66,123]. A good example is illustrated by Mingliag Fang and colleagues, who performed an interaction network analysis of lipidomics and metabolomics data to identify and select regulatory enzymes involved in metabolic pathways affected by triphenyl phosphate (TPhP). After following a molecular docking analysis a validation step was performed using biophysical and activity assays [124]. In another example, untargeted metabolomics was used to elucidate MoA and short-term response to antibiotics by the group of Sauer [125]. By monitoring almost 750 intracellular metabolites, they concluded that the metabolic changes were drug and dosage specific, and revealed a time-dependent sequence of events in which metabolism played a direct role in mediating the response to induced antibiotics stress towards cell death or escape. In a follow up study, the same group proposed a strategy to classify the MoA of antimicrobial compounds by performing rapid and systematic metabolic profiling [126]. The authors built a reference base of metabolic responses using 62 reference compounds whose modes of action in *Mycobacterium smegmatis* were well-known. Longitudinal analysis revealed significant differences between compounds targeting metabolism versus protein/DNA processing. Importantly, the so obtained metabolic profiles were of predictive value and allowed the authors to query the MoA of uncharacterized compounds. Müller et al. combined minimal inhibitory concentration testing and target elucidation for antifungal drugs in a single assay using a targeted GC/MS based approach [127]. Using this assay the authors successfully classified several novel antifungals and their target enzymes [128,129]. Examples in the field of neurodegenerative diseases include drug development for re-myelinating diseases as well as Alzheimer's disease (AD). In the case of de-myelinating neurodegenerative diseases, Najm et al. carried out a high-throughput chemical screening which identified small molecules causing oligodendrocyte formation and remyelination *in vivo*, a crucial

process in neurodegenerative diseases as for example multiple sclerosis [130]. After having identified phenotypic hits, the authors relied on a targeted GC/MS based metabolomics approach [131] to deduce the MoA of their screening hits as well as elucidate structural requirements of bioactive endogenous metabolites mediating the desired phenotype of oligodendrocyte formation and remyelination [132]. In another example, cholesterol metabolism was identified as a druggable axis in Alzheimers disease [133]. The authors used a phenotypic screen of FDA approved drugs to identify candidates, which subsequently underwent mechanistical investigations using metabolomics (lipidomics) approaches. In summary, it is evident from the aforementioned examples that metabolomics analysis can reveal detailed insights into drug induced metabolic alterations which can benefit the MoA and target elucidation of candidate drugs.

Limitations

Metabolomics shows great promise for drug discovery programs. However, it should be used in a dedicated, focused manner requiring awareness of possible pitfalls and limitations. The main challenges are i) large amounts of data, ii) difficult biological interpretation (missing pathways), iii) differentiating general from target specific metabolic alterations and iv) biologically linking drug induced PR to the target under investigation. For example, nuciferine, an alkaloid with unknown target was investigated in a high-fat diet induced non-alcoholic fatty liver rat model [134]. Nuciferine significantly influenced the disease phenotype as well as the metabolome/lipidome of the study animals. Based on untargeted metabolomics analysis the authors identified roughly twenty significantly altered serum metabolites from different pathways. While such data is very useful to further hypothesis generation it is very cumbersome if not impossible particularly in an in vivo setting to point out target and MoA of the applied treatment. To this end it seems impossible to distinguish if the observed metabolic changes are a primary or secondary effect of the treatment and which exact factors are underlying the observed phenotypic changes. Nevertheless, particularly stem cell based approaches might in the future allow to design metabolically "cleaner", but nonetheless disease relevant approaches allowing for a more facile correlation between drug induced metabolic alterations and disease phenotype. In other words, stem cell based systems as for example microtissues which can metabolically be controlled through the use of chemically defined media in combination with isotope tracers will allow to shed light on metabolic fluxes. Comparing, control, disease as well as treatment conditions should ultimately allow us to distinguish metabolic nodes under disease and treatment regulation.

Prediction of adverse effects (AE)

Subsequent to the *in vitro* evaluation of an NCE, preclinical and clinical studies are initiated. The outcome from these studies is expected to provide information on dose-dependent adverse effects, guidance on compound-specific monitoring at the clinical stage, as well as the pharmacokinetics and pharmacodynamics of the potential drug [135]. Metabolomics appears as an excellent option to track and evaluate a drug candidate's metabolism and side effects across the preclinical and clinical stages. This also stretches into the fields of personalized medicine and pharmacometabolomics, topics that have recently been reviewed elsewhere [136,137]. Recently, Wang et al. [138] and Griffin [139] have summarized the latest developments about the use of metabolomics for drug toxicity monitoring. Examples include the investigation of toxicity mechanisms of isoniazide and rifampicin [140], triptolide induced liver injury [141] or the application of NMR based metabolomics to monitor drug induced steatosis [93]. Besides these ongoing developments, metabolomics are recently also being used to investigate dietary influences on drug therapy. For example, Warth et al. monitored amino acid and central carbon metabolism of breast cancer cells under palbociclib and letrozole treatment in combination with xenostrogens [141]. Similar investigations form the basis for the investigation of drug-exposome interactions [142]. In summary, over the last two decades metabolomics has extensively been applied in pharmacometabolomics and the assessment of adverse drug effects. Nevertheless, novel fields such as drug-exposome interactions have recently started to evolve and might become an important part for personalized therapeutic guidelines.

Drug repurposing

Another upcoming area in which metabolomics methods are involved is drug repurposing. Drug repurposing has emerged as a new strategy investigating novel indications for registered drugs [143]. Metabolomics analysis appears to be an excellent choice for large screening campaigns in this field, allowing to biochemically characterize registered drugs and extrapolate the obtained data to known and relevant disease specific patho-physiological considerations. Practically, this approach involves the generation of large-scale metabolic databases mapping biochemical alterations under drug treatment to cell lines or to differentiated human-induced pluripotent stem cells (hiPSCs)-derived models, either cultured as a single cell type, or in the form of microtissues when combined with several cell types. Until recently, most databases mapping drug induced metabolic changes have mainly focused on proteomics, transcriptomics and genomics data [144]. However, Wages et al. recently mapped the effects of 1003 registered drugs on distal cholesterol biosynthesis [145]. Other examples stem from different disease areas such as parasitic

infections [146], cancer [147], depression [148] and Alzheimer's disease [149]. Recently, selected potential drug candidates were tested, targeting one carbon metabolism using isotopic tracing in combination with untargeted metabolomics. From this selection sertraline, a clinically used antidepressant, proved to target the serine/glycine biosynthesis pathway, and stands out as a good candidate adjuvant for cancer treatment in combination with mitochondrial inhibitors [150]. Moreover, sertraline also proved to exhibit leishmanicidal activity as shown by Lima et al. [151]. By combining metabolomics (LC-MS and CE-MS) with transmission electron microscopy the authors found a remarkable variation in thiol-redox and polyamine biosynthetic intermediates, as well as a shortage of intracellular amino acids under treatment with sertraline. This metabolic disarray correlated with anti-parasitic activity of the drug. Along these lines, statins, traditionally used as lipid-lowering agents, have shown promising anticancer effects, as determined by Kobayashi in 2017 who tested lovastatin on ovarian cancer cell lines and mice. A marked concentration-dependent inhibitory effect on tumor cell growth was observed, concluding that the anticancer effect of lovastatin was mostly associated to a reduction in the Warburg effect due to an augment in the TCA cycle and a reduction in lactate production by the cancer cells [152]. Simvastatin probed an antimicrobial effect on *Escherichia coli* activating energy metabolism, as well as amino acid, purine and pyrimidine anabolism [153]. Overall, metabolomics analysis in combination with advanced bioinformatics tools and pathway analysis shows great promise for advancing and potentially accelerating drug repurposing campaigns.

Concluding remarks and outlook

Novel phenotypic and genetic approaches introduce an increasing number of biochemically ill characterized targets into the drug development pipelines. However, sound biochemical knowledge of targets under investigation is mandatory for establishing useful TE markers and the understanding of the MoA. Metabolomics has started to evolve as a useful and effective tool for gathering these insights. Increasingly extensive targeted MS-based metabolomics platforms are capable to analyze hundreds to thousands of metabolites and thus aid in the course of deciphering TE, PR and MoA markers. These approaches are seconded by untargeted metabolomics analysis allowing to discover yet unknown metabolites. Moreover, untargeted approaches are particularly useful for PDD derived candidates where no prior MoA information is available. Additionally, yet unexplored or unexpected biochemical pathways might be discovered, shedding light not only on the MoA but also possible off-target and/or side-effects. Moreover, NMR based

metabolomics has emerged as an ideal platform to study abundant metabolites (e.g., central energy metabolism) in a quantitative fashion and remains a solid option for isotope tracing and MFA, complementary to established MS methods. Next, to the application of metabolomics in TE and MoA-based research, metabolomics analysis is now becoming established in pharmacometabolomics and pre-clinical drug toxicity investigations. Moreover, new fields such as drug-exposome interactions start to evolve. Finally, drug repurposing might significantly benefit from a deep understanding of drug induced metabolic alterations combined with multi-omics integration, advanced bioinformatics tools and pathway mapping.

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Declaration of interest

There are no conflicts of interest or disclosures associated with this manuscript.

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