Current and future approaches for *in vitro* hit discovery in diabetes mellitus

Xin Zhang [a], Erwin Kupczyk [a,b], Philippe Schmitt-Kopplin [a,b,*], Constanze Mueller [a,*]

[a] Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München, Ingolstaedter Landstr. 1, 85764 Neuherberg, Germany
[b] Comprehensive Foodomics Platform, Chair of Analytical Food Chemistry, TUM School of Life Sciences, Technical University of Munich, Maximus-von-Imhof-Forum 2, 85354 Freising, Germany

Type 2 diabetes mellitus (T2DM) is a serious public health problem. In this review, we discuss current and promising future drugs, targets, *in vitro* assays and emerging omics technologies in T2DM. Importantly, we open the perspective to image-based high-content screening (HCS), with the focus of combining it with metabolomics or lipidomics. HCS has become a strong technology in phenotypic screens because it allows comprehensive screening for the cell-modulatory activity of small molecules. Metabolomics and lipidomics screen for perturbations at the molecular level. The combination of these data-intensive comprehensive technologies is enabled by the rapid development of artificial intelligence. It promises a deep cellular and molecular phenotyping directly linked to chemical information about the applied drug candidates or complex mixtures.

Keywords: Cell painting; High-content screening; Functional metabolomics; Lipidomics; Bioactives; Drug discovery

Introduction

Diabetes mellitus (DM) is a chronic metabolic disease characterized by persistent hyperglycemia. According to the International Diabetes Federation (IDF), DM caused 6.7 million deaths in 2021, and the global DM incidence is predicted to increase 68% by 2045.[1] Among the main types of DM: Type 1, Type 2 and gestational DM, Type-2 diabetes mellitus (T2DM) is the most dominant form, accounting for ~90% of the cases worldwide. It is characterized by insulin resistance and β-cell dysfunction with genetic factors, sedentary lifestyle and environmental risks being major predisposing factors. Furthermore, T2DM, with its comorbidities of obesity and inflammation, forms a complex pathophysiological network associated with many severe complications,[2] and management is extremely difficult. Current therapies such as insulin injections and oral hypoglycemic agents mainly augment insulin-mediated glucose disposal. Molecular drugs successfully reduce hyperglycemia and are inadequate to address the multifaceted pathophysiology of T2DM and its comorbidities resulting in a lack of efficiency. Considering the prevalence of limited therapeutic options for T2DM, the need to develop novel drugs is urgent. All drug development requires robust high-throughput (HTP) bioactivity assays that systematically evaluate the biological response of candidate molecules. Historically, *in vivo* assays dominated T2DM research because they provide rich information on compound efficacy and toxicity. An overview of numerous animal models is given by Kleinert and colleagues.[3] Owing to ethical issues, nowadays *in vitro* assays are increasingly popular. This development is in line with stricter political regulations and it reflects the political will to reduce animal testing. Accordingly, the EU parliament has begun to ask for a reduction in animal experiments.[4] Excluding the ethical issue, *in vivo* approaches are often costly and time-consuming compared with *in vitro* HTP screens. Therefore, we summarize current *in vitro* assays and give perspective to future

* *Corresponding authors at: Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München, Ingolstaedter Landstr. 1, 85764 Neuherberg, Germany (P. Schmitt-Kopplin). Schmitt-Kopplin, P. (schmitt-kopplin@helmholtz-muenchen.de), Mueller, C. (constanze.mueller@helmholtz-muenchen.de).*

more unbiased and comprehensive screening opportunities. Yet, the development of appropriate *in vitro* techniques is not a trivial task. It requires an understanding of parameters and pathways involved, especially in a multifaceted disease such as T2DM. Remarkable advances have been achieved in the landscape of biochemical and analytical chemistry, and integrated multidisciplinary approaches are available to study the pathophysiology of complex diseases.[5–7] Artificial intelligence (AI) is developing exponentially and aids the integration of data derived from orthogonal screens like high-content screening (HCS) and different omics methods to successfully characterize phenotypes in-depth,[8] preventing rediscoveries[9–12] and aiding the fundamental understanding of the underlying pathophysiological network and possible pathway crosstalk.[5,7,13,14] This review provides an overview of approved drugs, putative future targets and the associated glucose homeostasis, *in vitro* technologies for T2DM and future perspectives in comprehensive testing methods.

Approved drugs and future pathways of interest

To develop suitable and powerful *in vitro* screens, it is essential to understand molecular mechanisms and the targets involved. Therefore, we give a short overview of glucose homeostasis in T2DM (Fig. 1a). Because energy homeostasis is a very complex process, only the points that are essential for understanding

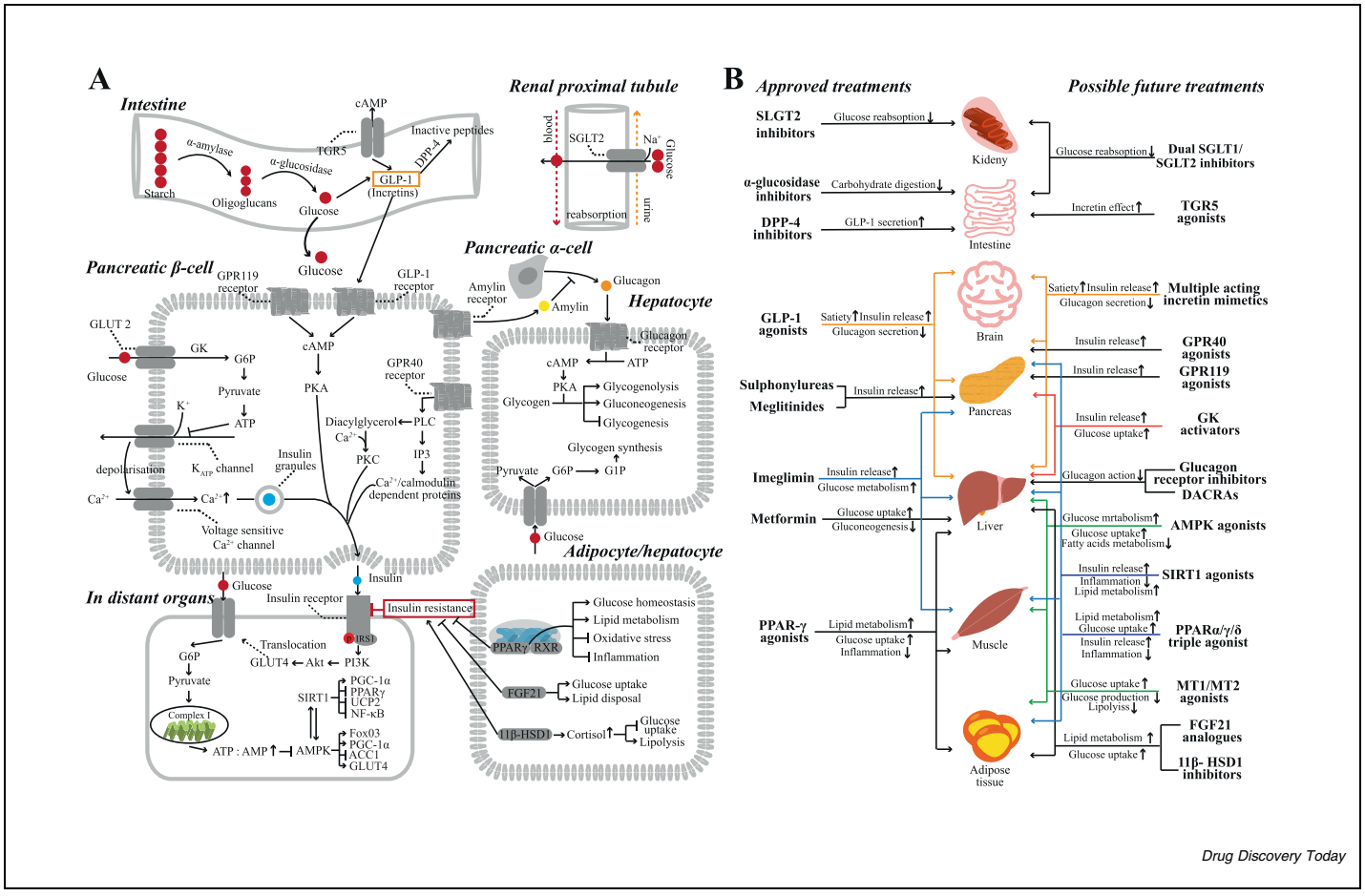


FIGURE 1

Overview of glucose homeostasis and treatments for T2DM. **(a)** Glucose homeostasis is achieved through the coordinated work of different systems. In pancreatic β-cells, glucose from food digestion undergoes glycolysis and increases intracellular ATP levels, which stimulates insulin release by closing ATP-dependent K^+ channels. Generation of cAMP and other cellular intermediates enhances glucose-induced insulin secretion with the involvement of incretin hormones, such as GLP-1, and the activation of several GPR. The pancreas can also release glucagon, which manages glucose homeostasis by counteracting insulin. Released insulin binds to the insulin receptor in distant organs, leading to the activation of PI3K. This subsequently increases glucose uptake via enhanced translocation of GLUT4. During this process, insulin resistance impairs the action of insulin and leads to aberrant glucose metabolism. Furthermore, renal glucose reabsorption also plays an important part in blood glucose regulation. **(b)** Approved treatments (left), possible future treatment (right) and their intervention sites. Insulin and its related treatments are not included. For detailed information like compound names belonging to each class, current stages of clinical phase and MOA, please refer to Tables S1 and S2 (see supplementary material online). Abbreviations: TGR5, G-protein-coupled bile acid receptor 1; cAMP, cyclic adenosine monophosphate; GLP-1, glucagon-like peptide-1; SGLT2, sodium-dependent glucose co-transporter 2; GLUT 2/4, glucose transporter type 2/4; GK, glucokinase; G6P, glucose 6-phosphate; ATP, adenosine triphosphate; AMP, adenosine monophosphate; PKA, protein kinase A; PLC, phospholipase C; IP3, inositol 1,4,5, trisphosphate; G1P, glucose 1-phosphate; AMPK, 5′ adenosine monophosphate-activated protein kinase; SIRT1, Sirtuin 1; FoxO3, Forkhead box O3; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1alpha; ACC1, acetyl-CoA carboxylase 1; NF-κB, nuclear factor-κB; FoxO1, Forkhead box O1; UCP2, uncoupling protein 2; PPARγ, peroxisome proliferator-activated receptor γ; G1P, glucose 1-phosphate; Akt, Akt kinase; PI3K, phosphoinositide 3-kinases; FGF-21, fibroblast growth factor 21; 11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; DPP-4, dipeptidyl peptidase-4 inhibitors; GPR40, G-protein-coupled receptor 40; DACRAs, dual amylin and calcitonin receptor agonists; GPR119, G-protein-coupled receptor 119; MT1/MT2, melatonin receptor 1/melatonin receptor 2.

the drug targets are highlighted. For a more detailed and comprehensive overview of the pathogenesis of T2DM, we recommend DeFronzo *et al.* and Röder *et al.* for further reading.[15,16]

Glucose homeostasis is achieved through the coordinated function of different organs, of which the pancreas plays a key part by releasing insulin and its antagonist glucagon. After their nutritional intake, complex polysaccharides are degraded in the gastrointestinal tract (GIT) with the help of α-amylases and α-glucosidases. After absorption, glucose is then transported to the distant organs, taken up and metabolized by glycolysis leading to increased pyruvate levels and, as a final consequence, elevated ATP production. The uptake of glucose in the cytoplasm is facilitated by glucose transporter type 4 (GLUT4), for which the translocation into the plasma membrane is insulin dependent. The required insulin is released by pancreatic β-cells, in which, upon glucose uptake and following increased ATP levels, ATP-dependent K^+ channels are closed, causing membrane depolarization and opening of Ca^{2+} channels. This ultimately leads to the exocytosis of insulin – this whole process is known as glucose-induced insulin secretion (GIIS).[17] Released insulin is the main hormone for glucose disposal in the body and is therefore essential to maintain the energy homeostasis. Pancreatic β-cells express another isoform of glucose transporter: glucose transporter type 2 (GLUT2), which is an insulin-independent bidirectional transporter and which functions as a gauge for serum glucose levels. In distant organs, such as muscle or adipose tissue, insulin binds to insulin receptors and activates the PI3K/Akt pathway, thereby promoting GLUT4 translocation and increasing glucose uptake.[18] Glucose is thereafter under aerobic conditions metabolized. Phosphorylation to glucose-6-phosphate (G6P) is hereby the first step, which is catalyzed by hexokinases with different isoforms according to the organ, followed by all steps through the glycolysis or pentose phosphate pathways and the citrate cycle and, finally, fully oxidized to carbon dioxide and water. These catabolic reactions lead to an energy yield, mostly in the form of ATP.

Importantly, there is also a gastrointestinal response to glucose metabolism. The GIT has evolved several regulatory pathways to manage glucose disposal and to influence insulin release. These are collectively referred as gastrointestinal-mediated glucose metabolism (GIGD) and include hormones, microbiota and first-pass hepatic glucose uptake. The ingestion of food and absorption of nutrients is associated with an increased secretion of GIT peptide hormones that act on distant organs. The incretin hormones, glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), which are secreted by enteroendocrine cells, are identified as the principal modulators of gut-mediated glucose homeostasis. Moreover, GLP-1 can stimulate insulin release by activating the cAMP/PKA and PLC/PKC pathways.[19,20] Activation of the G-protein-coupled receptor 119 (GPR119), a GPR mainly expressed in the pancreas and GIT, also increases GSIS via stimulating the cAMP/PKA signaling pathway.[21] Furthermore, free fatty acids (FFAs) can potentiate insulin release through the PLC/PKC pathway. When binding to GPR40, long-chain FFAs activate phospholipase C (PLC) and produce diacylglycerols (DAGs) and inositol-trisphosphate (IP_3). DAGs can activate protein kinase C (PKC) together with calcium, increasing insulin release. IP_3 stimulates the release of Ca^{2+} from the endoplasmic reticulum (Fig. 1a).[22,23].

Glucagon is another key hormone released by the pancreas that maintains glucose homeostasis by increasing blood glucose levels. It promotes hepatic glucose production by increasing glycogenolysis (the breakdown of glycogen) and gluconeogenesis (the generation of glucose from certain non-carbohydrate carbon substrates) and decreasing glycogenesis (synthesis of glycogen). By blocking glucagon action, glucagon receptor antagonists and amylin–calcitonin receptor agonists (DACRAs) exert hypoglycemic effects in T2DM.[24,25]

Further gut enzymes, gut hormones and various other gut-derived factors exert pleiotropic effects at different targets to regulate energy and glucose homeostasis. We recommend Holst *et al.* for the interested reader.[26] In Fig. 1a, we highlight GLP-1 because it is a good example for multifaceted metabolic effects in regulating glucose homeostasis. In addition to stimulating insulin release from pancreatic β-cells as described above, GLP-1 can inhibit food intake, delay gastric emptying and suppress glucagon secretion. Therefore, GLP-1-targeted therapies including GLP-1 mimetics and incretin enhancers (DPP-4 inhibitors, dipeptidyl peptidase 4-inhibitors) have been used to treat T2DM. Meanwhile, multi-acting incretin-mimetics and treatments targeting G protein-coupled bile acid receptor 1 (TGR5) are under development because of the regulatory role of TGR5 in GLP-1 release.[27,28].

In addition, 5′-adenosine monophosphate-activated protein kinase (AMPK) and Sirtuin 1 (SIRT1) are two important enzymes that are involved in the regulation of glucose and lipid metabolism; and are closely related to the pathogenesis of insulin resistance. AMPK can be understood as a sensor of AMP:ATP or ADP:ATP ratios. Its activation can regulate the expression of various genes involved in energy metabolism, inhibiting anabolic processes that consume ATP (such as gluconeogenesis) and the activation of catabolic processes that produce ATP (e.g., fatty acid uptake and oxidation).[29] Activation of AMPK can also promote GLUT4 translocation and consequently stimulates glucose transport.[30] Coordinately, SIRT1 functions to maintain glucose homeostasis and promote insulin release. SIRT1 can also regulate glucose and lipid metabolism by acting on peroxisome proliferator-activated receptor γ (PPARγ), thereby improving insulin resistance.[31] PPARs regulate the expression of many genes involved in energy metabolism, adipogenesis and inflammation. Among them, PPARγ is most closely associated with T2DM because of its link to insulin sensitization. Upon activation, PPARγ forms heterodimers with the retinoid X receptor (RXR) that regulate transcription of genes involved in lipid metabolism, glucose homeostasis, oxidative stress and inflammation.[32]

Tubular glucose reabsorption, renal glucose uptake and renal gluconeogenesis are involved in regulating glucose homeostasis. Increasing urinary glucose excretion and decreasing the blood glucose level in an insulin-independent manner by inhibition of sodium glucose co-transporter 2 (SGLT2) via SGLT2 inhibitors used therapeutically.[33]

All the above pathways contain drug targets or targets for drugs under development (Fig. 1b). Further details are reviewed

by Clapham *et al.* and Chaudhury and co-workers.[34,35] Overall, 36 oral drugs out of eight classes are currently being used for the treatment of T2DM (see Table S1 in the supplementary material online). In addition, two first-in-class medicines have been recently approved, one is imeglimin hydrochloride, which advances glucose metabolism in the liver and skeletal muscle by acting on mitochondria and promoting GSIS.[36] The other is chiglitazar, a non-thiazolidinedione PPARα/γ/δ triple agonist with favorable effects on reducing lipid accumulation, anti-inflammation and lowering blood glucose levels.[37]

Metformin is the first-line oral antidiabetic drug worldwide. It enhances insulin sensitivity, increases the hepatic uptake of glucose and inhibits gluconeogenesis by inhibiting mitochondrial complex I and indirectly activating AMPK in the liver. It is the medication of choice in overweight patients owing to appetite reduction, caused by a stimulation of growth/differentiation factor 15 (GDF15) secretion.[38] Additional T2DM medications act by reducing the absorption of sugars from the intestines (α-glucosidase inhibitors), by the inhibition of the enzyme DPP-4 (DPP-4 inhibitors), by enhancing the incretin effect of GLP-1 (GLP-1 agonists), by a sensitization of the body to insulin (thiazolidinediones, PPARγ agonists), by a raised insulin release through blocking ATP-dependent K^+ channel (sulfonylureas, meglitinides) or by increasing the sugar excretion into the urine (SLGT inhibitors)[39] (Fig. 1b; Table S1 online). After ingestion of food, a simple way to limit glucose uptake is nutrient restriction by α-glucosidase inhibitors, limiting blood glucose levels by blocking the hydrolysis of dietary carbohydrates into monosaccharides such as glucose. Thiazolidinediones (PPARγ agonists) ameliorate insulin resistance, decrease lipolysis and show anti-inflammatory effects. Sulfonylureas and meglitinides block the ATP-dependent K^+ channel and stimulate insulin secretion. SGLT2 inhibitors lower blood glucose levels by inhibiting the reabsorption in the kidney. Furthermore, additionally marketed antidiabetic drugs including pramlintide, an amylin analog, or bromocriptine, a dopamine D2 receptor agonist, are of minor importance owing to their frequency of application or indications for complications in T2DM.

Next to the above-mentioned approved drugs, 35 novel antidiabetic drugs are in late-stage clinical trials.[40] Details about the candidates and their targets are given in Fig. 1b and Table S2 (see supplementary material online). Overall, they can be classified by three main modes of action (MOA): stimulate insulin secretion, improve insulin resistance and modulate glucose homeostasis. Dorzagliatin and tirzepatide are two first-in-class insulin secretagogues that have completed Phase III clinical studies. Dorzagliatin is a dual glucokinase (GK, the present isoform of hexokinase IV in the pancreas and liver) activator that induces GSIS and increases glucose uptake by activating GK in pancreatic β-cells and the liver.[17] Tirzepatide is a novel incretin mimetic that promotes insulin release by simultaneously activating GLP-1 and GIP.[41] AMPK has also attracted attention as a potential therapeutic target owing to its energy-sensing functionality. Although several marketed drugs, such as metformin, indirectly activate AMPK, there is no direct AMPK activator available on the market. Another target of essential importance in the regulating of cellular metabolism is Akt [also known as protein kinase B (PKB)].[42] Convincing evidence proves that Akt signaling is associated with insulin resistance mainly through stimulation of translocation of GLUT4, inhibition of glycogen synthase kinase 3 (GSK-3) and phosphorylation of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α). As aforementioned, another enzyme involved in the regulation of glucose metabolism is SIRT1 which acts through its deacetylase activity. Activation of SIRT1 in pancreatic β-cells promotes insulin secretion and influences oxidative stress and inflammation. Other promising therapeutic targets that stimulate insulin secretion are GPR119 and GPR40. GPR119 is mainly expressed in the pancreas and GIT. Its activation stimulates insulin secretion by increasing cAMP levels, and it promotes the release of gut peptides such as GLP-1 and GIP.[43] GPR40 [also termed free fatty acid (FFA) receptor-1] is mainly expressed in pancreatic β-cells and its activation induces FFA-stimulated insulin secretion. Moreover, selective agonists targeting GPR40, which are expressed in intestinal L and K cells, stimulate the secretion of GLP-1 and GIP, which eventually promote insulin secretion.

In addition, novel therapeutic targets aimed at improving insulin sensitivity can be of great benefit for glycemic control in T2DM. For example, 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) is a cortisone reductase and its inhibition decreases the intracellular level of cortisol, which inhibits adipogenesis and improves insulin sensitivity.[44] Fibroblast growth factor 21 (FGF-21) is a hormone that can normalize glucose and lipid metabolism by reducing lipolysis and rising glucose uptake.[45] Melatonin and the activation of melatonin receptor (MT1/MT2) can also improve insulin sensitivity by regulating multiple metabolic processes such as glucose production and lipolysis.[46].

Furthermore, the use of compounds that attack multiple targets and thus have synergistic effects seems to be a promising strategy for T2DM.[47,48] Therefore, among the new drug candidates are dual- or triple-action compounds. Importantly, such synergistic-acting drugs are considered very effective and tolerable with fewer side effects. They interact with the pathophysiological network at different targets (poly-pharmacology) to improve the therapeutic outcome and reduce required doses compared with single-action compounds.[47–50] Phytopharmaceuticals that have naturally a poly-pharmacological effect are an additional supportive therapy option and are frequently discussed as a source of natural products with antidiabetic characteristics. Considering the multifaceted pathophysiology of T2DM, pleiotropic effects of key players in the glucose metabolism and tremendous pathway crosstalk of the above presented targets, comprehensive *in vitro* approaches are inevitable in antidiabetic research.

Traditional in vitro assays for T2DM

In general, drug screens follow two main strategies: target-based or phenotypic screens.[51] Target-based methods rely on the precise knowledge of the drug target; therefore, their biggest advantage is that they directly give a precise MOA. A major drawback of target-based assays is the lack of ability to detect off-target or secondary effects on the poly-pharmacological network. Such a phenomenon of one molecule unintentionally interacting with multiple targets is very common and results in side effects. It is often discovered at a later stage during drug discovery and limits success rates. Nevertheless, a controlled poly-pharmacology can also offer

a unique opportunity for therapeutic intervention in multifaceted diseases such as T2DM, as aforementioned.[52] Currently, enzyme inhibition assays, which screen in a target-based manner, are the most common way to discover antidiabetic compounds. For example, inhibition assays of carbohydrate-digesting enzymes (such as α-amylase and α-glucosidase) offer an effective strategy for compound screens with a putative antidiabetic effect. Other assays target protein tyrosine phosphatase-1B (PTP-1B), DPP-4, GLP-1 or PPARγ (Box 1).

Phenotypic screens, as opposed to targeted, offer the ability to search for a certain phenotype regardless the underlying MOA or poly-pharmacological crosstalk; target deconvolution should be performed thereafter to clarify the MOA. Classic T2DM phenotypic assays are glucose uptake assay, reporter gene assay, insulin secretion assay, calcium measurement assay, ATP measurement assay and cAMP assay (Box 1).

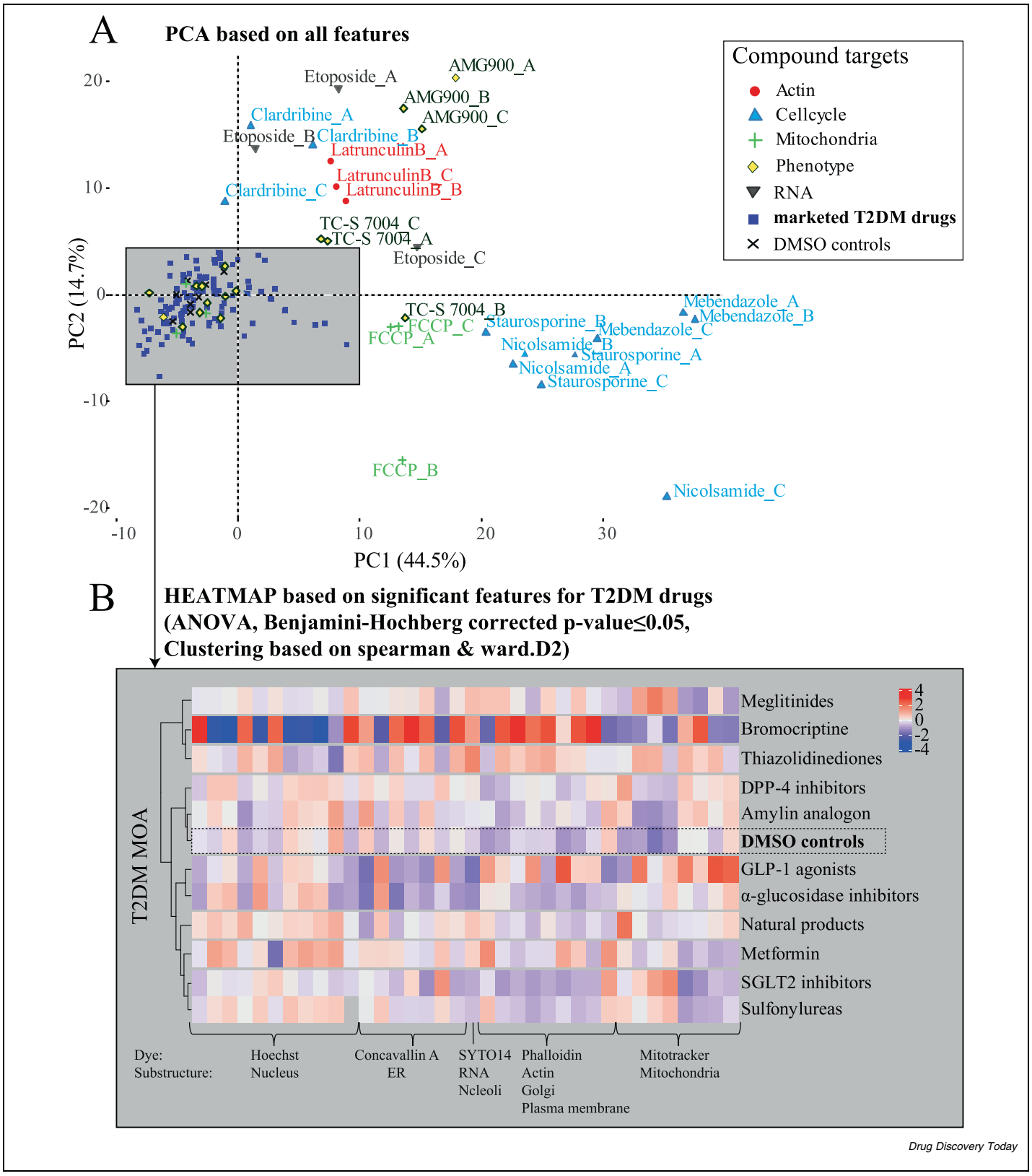
During the past few years, image-based phenotypic screens have reshaped the traditional drug discovery process. Today, one of the most promising new phenotypic screening technologies is HCS. Cell-based HCS uses automated HTP fluorescence microscopy to capture images with ultrastructural resolution. Once images are acquired, many cellular or morphological features and properties are extracted and used for comprehensive phenotypic analysis on a single-cell level.[53] This ability to reveal and quantify many features and properties simultaneously has made HCS broadly accepted in the pharmaceutical industry and academia. In particular, a protocol published by Bray *et al.* that uses six fluorescence dyes to capture the morphology of eight intracellular organelles is widely applied and termed 'cell painting'.[54] Several expansions or modified protocols are additionally implemented. For instance, Kremb *et al.* and Kang *et al.* presented screens targeting more organelles or focusing on functional aspects.[8,55] Further hybrid approaches might use modified cells, further antibodies or focus on a set of specific disease-related targets.[56] Likewise, Hill *et al.* studied the differentiation state, survival and/or proliferation of pancreatic β-cells upon treatment with extracts isolated from marine invertebrates using HCS. To the best of our knowledge, this is the first implementation of multiparameter HCS published for diabetes research. They analyzed insulin promoter activity, pancreatic and duodenal homeobox 1 (pdx1) promoter activity, nuclear morphology and cell number in parallel, with insulin being the primary parameter to evaluate the fitness and maturity of pancreatic β-cells. pdx1 was used as master β-cell transcription factor, controlling the survival and function. Nuclear morphology and cell number assessed apoptosis and cell viability. The analysis was performed in human islet cells and MIN6 cells.[57] Furthermore, Wolff *et al.* studied the nucleus-to-cytoplasm translocation of GK. Glucokinase regulatory protein (GKRP) reduces the metabolic activity of the enzyme GK as it locks it in an inactive state within the nucleus. The authors used primary rat hepatocytes as the model cell system, because they endogenously express GK and GKRP. The (semi-)automated screening platform enabled profiling of allosteric activation of GK for several compounds.[58].

A remarkable advantage of HCS is its independence from a certain MOA or target, because the readout simply reflects any

Box 1 Summary of frequently applied *in vitro* assays in T2DM research.

Target-based screens	**α-Amylase and α-glucosidase assay:** the activity of α-amylase and α-glucosidase can be determined colorimetrically using their respective substrates. For α-amylase, a fluorescence-based biochemical assay can also be used. In addition, chromatographic methods, such as ligand fishing and affinity ultrafiltration-HPLC-MS, have been developed for rapid *in vitro* screens of α-glucosidase inhibitors. **GLP-1 receptor and PPAR-γ assay:** the receptor-binding assay and β-arrestin recruitment assay can be used to screen for GLP-1 receptor agonists, and PPAR-γ transactivation assays are commonly used for screening for PPAR-γ agonists. **PTP-1B and DPP-4 assay:** *in vitro* activity of PTP-1B and DPP-4 are also commonly evaluated with colorimetric and fluorometric assays and these assays are usually performed with recombinant enzymes. Commercial kits for PTP-1B and DDP-4 activity are also available. In addition, MS-based screening for DPP-4 inhibitors has been established. **GLUT4 translocation assay:** classical GLUT4 translocation assays use western blotting, immunofluorescence and qPCR to detect GLUT4 protein and gene expression. Novel imaging techniques, such as TIRF microscopy and ultrafast microscopy, allow visualization of GLUT4 translocation.
Phenotypic screens	**Signaling pathway assays:** signaling pathway assays include assays such as protein–protein interaction and gene reporter, and asses the effect of drug candidates on key components of one or multiple T2DM-related signaling pathway. Compared with target-based screening methods, signaling pathway assays can identify compounds acting at different targets (known or unknown) in the signaling pathways. **T2DM-like phenotypic assays:** T2DM is characterized by hyperglycemia, insulin deficiency and insulin resistance. Assays that provide readouts about these T2DM-related characteristics can be used to screen for anti-T2DM compounds. For example, glucose uptake can be measured with radioisotope methods to capture potential insulin sensitizers; measurements of intracellular calcium, insulin, cAMP and ATP can determine compound effect on insulin secretion and help find potential insulin sensitizer. The glucose uptake assay is the most basic screening assay for T2DM. Glucose uptake can be analyzed with radioisotope methods using 3MG and 2DG as radiolabeled glucose analogs. An enzymatic, fluorometric resazurin assay is furthermore frequently applied to measure the glucose uptake in microplates. Insulin secretion assays are essential in T2DM research. They are applied to investigate causes of hypoglycemia, assess β-cell function and screen insulin secretagogues. Intracellular insulin levels are commonly analyzed by ELISA-based assays and radioimmunoassays. Ca^{2+} is an important second massager and participates in the regulation of several cellular processes such as insulin secretion. Intracellular Ca^{2+} concentration is analyzed with fluorescent Ca^{2+} indicators. Intracellular cAMP and ATP levels have a direct influence on insulin secretion. Intracellular cAMP can be measured with radioimmunoassays and FRET-based assays, whereas ATP levels can be measured with a luminescence-based assay. In addition, ELISA kits are available to measure cAMP and ATP levels.

phenotype induced by a substance. Therefore, the phenotype based on a poly-pharmacological network can also be reflected and studied. Such a phenotype can be either caused by a drug candidate during hit screening or it can be intentionally induced to study further effects on the poly-pathophysiological network.[10–12] However, usually a library of reference phenotypes is first built as landmarks. The main compounds of all classes of approved antidiabetic drugs cause measurable phenotype modifications using the standard cell painting protocol, enabling screening of comparable phenotypes (Fig. 2). It is important to



mention that cell painting is mentioned here as a representative for multiparameter HCS. The reason is the advantage of a standardized protocol that can be exchanged between laboratories worldwide. Of course, the concept can be applied to other multiplexed HCSs.

Furthermore, important to note is that HCS is compatible to many cell lines, primary cells, stem cells and even organoids.[59] Standard cell lines applied in cell painting are U2OS and A549. Typical antidiabetic model cell systems are given in Table S3 (see supplementary material online), with 3 T3-L1 (mouse fibroblast), L6 (rat myoblasts), C2C12 (mouse myoblasts), HepG2 (human hepatic carcinoma) and human liver cells being the most common ones. In addition, pancreas organoids have become a promising tool for *in vitro* diabetic research.[60] The feasibility of applying HCS in these 3D culture models is worth exploring and might be a crucial step toward translation of the *in vitro* results. Anyway, the use of diverse cell types to comprehensively evaluate the potential of chemicals is highly recommended.[61] HCS performed with a disease-model cell-line enables signature-based screens by measuring which compounds can reverse disease-dysregulated pathways and related phenotypes (so called reprogramming).[62] Interestingly, apart from the above-mentioned screen by Hill *et al.*, we found no further research using a multiparameter cell painting platform for the screening of antidiabetic drugs. Nevertheless, the polypharmacology of T2DM in particular makes cell painting a promising technology because it provides a direct and comprehensive information on compound-induced perturbations on multiple levels of cell physiology, multi-level toxicity, potential molecular targets and off-target effects, as well as SAR.[63]

Owing to the sheer amount and complexity of data generated via cell painting, computational analysis is key to the success of such screens. A typical data analysis workflow runs from high-throughput imaging to feature extraction (e.g., image analysis and quality control) and data preparation (e.g., correction for batch and plate-layout effects, normalization and transformation), before the data are visualized and statistically analyzed.[64] The biological activity and MOA of the compounds of interest are predicted based on the analysis of compounds with similar biological activity and that therefore share a comparable phenotypic profiling.[65] In this process, machine learning (ML) strategies, including unsupervised and supervised methods, are frequently applied. Unsupervised ML strategies are well suited to recognize hidden patterns in the data, cluster the data based on similarities or to represent the data in a lower dimensional space. Commonly used unsupervised ML algorithms for these tasks are principal component analysis (PCA), uniform manifold approximation and projection (UMAP), t-distributed stochastic neighbor embedding (tSNE) and hierarchical clustering. Likewise, unsupervised ML is successfully used in phenotypic screens by helping to identify biological similarities, such as MOA or SAR.[66] Unsupervised ML has a big advantage in that no prior knowledge of the data is needed. By contrast, supervised ML requires prior knowledge. The algorithms of supervised ML are trained with predefined labels to be able to make predictions on unknown data. The labeling of the data makes supervised methods more laborious and costly than unsupervised methods. But this allows the algorithms to perform specific tasks, such as regression or classification. Support vector machines (SVM) and artificial neural networks (ANN) are often used for classification problems in HCS.[67–69] SVM are algorithms used for regression or classification and are therefore applied to classify morphological differences,[71,72] different MOA[72] or the MOA.[70,73–81] SVM adjusts a hyperplane in the feature space that separates the classes in the best way. The algorithm adjusts the hyperplane so that the distance of the objects closest to the plane is maximized. ANN are also popular algorithms in HCS and are often used for feature extraction.[76,81] They have very flexible architectures, because the number of layers, the number of neurons in the layers and the type of connection between the layers can be varied. This gives a huge freedom to adapt the architecture of an ANN to the task. A neural network consists of an input, one to several hidden layers and an output layer. Each node in a layer is connected to several nodes in a lower layer and these connections are differently weighted. These weights must be learned by the neural network. If the output of a node exceeds a certain threshold, it is forwarded to the lower layers. In this way, the inputs are sent through the whole network and in the end generate the output. In summary, unsupervised methods are used to categorize the data or to reduce the dimensions of the data. Supervised ML is used to perform specific tasks such as regression or classification. A major drawback for unsupervised machine learning models is their lack in recognition of new and unknown patterns, as the models force the data in previously defined categories. One way to tackle this issue is to use novel detection. Unsupervised ML does not require labeled data.

Metabolomics and lipidomics

Metabolomics refers to the HTP comprehensive analysis of all small molecules (up to a molecular mass of 1500 Da) in biological samples, in comparison lipidomics targets lipids.[82,83] Owing to the manifold biological functions of single metabolites and the

FIGURE 2

Cell painting profiles of marketed T2DM drugs, reference chemicals and DMSO controls in U2OS cells. **(a)** In the principal component analysis (PC1 vs PC2) it is shown that T2DM-marketed drugs (navy blue squares) elicited mild phenotypic responses compared with compounds that have a strong phenotypic effect (red circles: chemicals affect polymerization of actin; blue triangles: chemicals disturb cell cycle; green crosses: chemicals disrupt mitochondrial function; yellow diamonds: chemicals change cell phenotype; dark green triangles: chemicals cause DNA damage; black crosses: DMSO controls). Each compound was measured as a biological triplicate, each in ~1000 cells. 164 features were extracted from raw images. The PCA was generated using all 164 measured cellular features. **(b)** Heatmap for features that are significantly different comparing classes of T2DM drugs with DMSO controls. A Kruskal-Wallis rank sum test was performed and P-values were corrected for multiple testing using Benjamini and Hochberg as the adjustment method. Significance cut-off was set at P-value $\leq$ 0.05. The number of significant features (72 out of 164) was further reduced by omitting highly correlated (>0.95) features. Data are shown as mean per class. Each compound was measured as a biological triplicate, each in ~1000 cells. Colors indicate the scale of increase (red) or decrease (blue) in phenotypic features with respect to DMSO controls. For a detailed method description, please see supplementary material method section online.

complex crosstalk between metabolic pathways, metabolomics research offers a broad scope as a phenotypic screen on a molecular level. Direct information on the cellular response to variations in the internal and external environment can be accessed and facilitate a deeper understanding of the underlying pathophysiology.[84] In metabolomics studies, either the discovery of perturbation of the small-molecule inventory of a cell or organism itself and related biomarkers can be a focus (so called discovery metabolomics)[5,13,82,85] or the study can aim for a more systematic understanding of the mechanisms and possibilities to reprogram the cell or system (so-called functional or reprogramming metabolomics).[86–88] Alternatively the identification of bioactive small molecules is the focus (so-called activity metabolomics).[86,89,90] Currently, discovery metabolomics is the most common application, but hunting for mechanisms and reprogramming possibilities by functional metabolomics is particularly promising for studying of prevention and treatment opportunities of multi-pathological disease.[87,88] Importantly, because most traditional drugs are below a mass of 500 Da (Lipinski's Rule of Five), activity metabolomics has become an important tool in the search for novel bioactive substances and hit discovery. A very interesting review about recent advances in metabolomics in early drug discovery has been published by Alarcon-Barrera and colleagues.[84] Finally, metabolomics tools for enhanced structure identification and early recognition of rediscoveries (e.g., by molecular networking) can successfully aid the drug discovery process.[9]

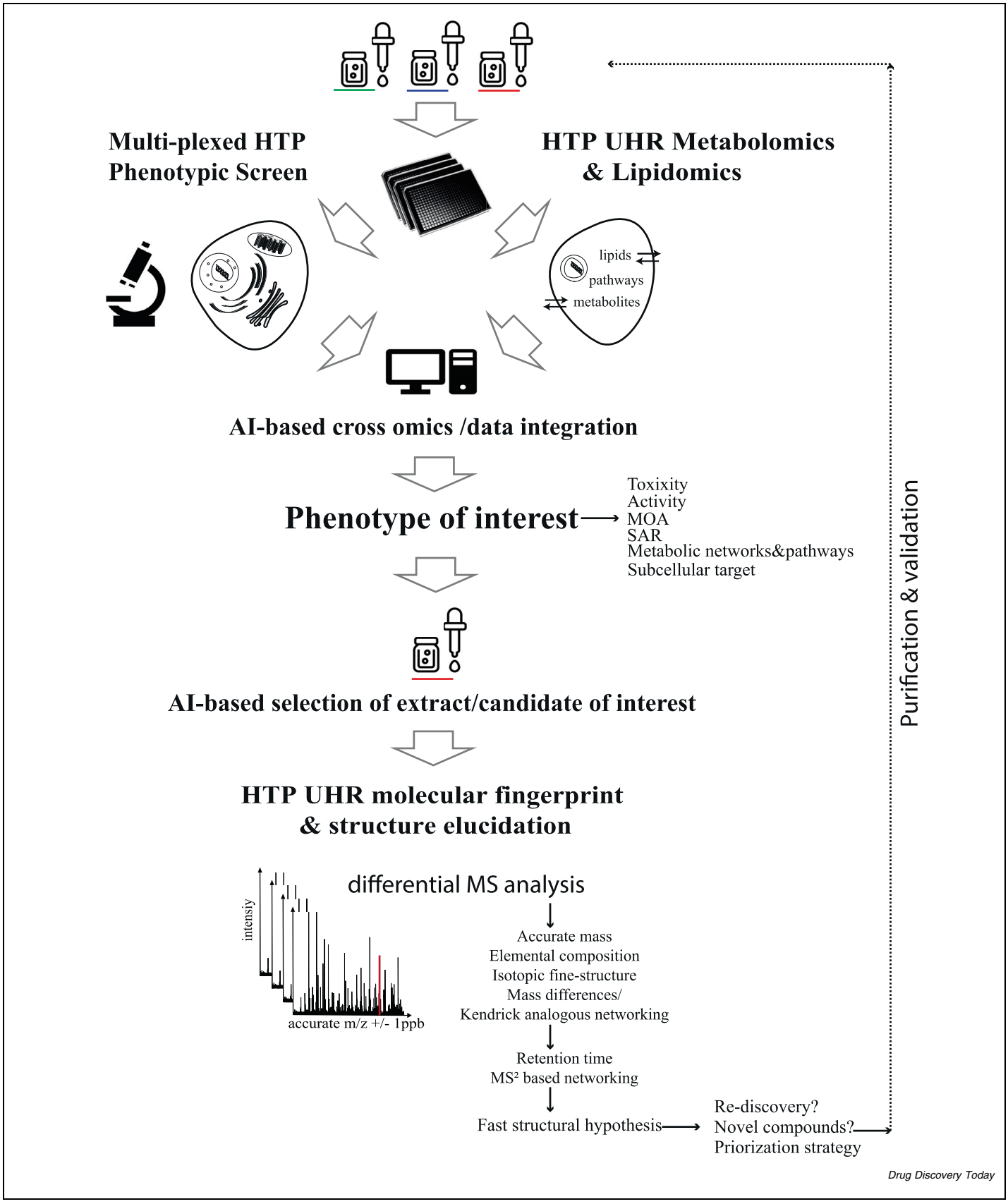
Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the key technologies in metabolomics. The latter is either in direct-injection mode or hyphened to a separation technique such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE). MS provides a mass:charge ratio for each analyte as well as its relative abundance in a mixture of molecules. It is therefore a semiquantitative method. To identify compounds, the selected analyte ions can be allowed to collide with either gas (collision-induced dissociation) or electrons (electron-capture dissociation) to form product-ions indicative for the chemical structure of the parent-ion – a technique known as MS^2. The aforementioned coupling with chromatography techniques can be applied to select compound classes of interest, reduce matrix effects and add another dimension to compound identification.[91] In contrast to MS, NMR determines chemical structure by using nuclear spins in a magnetic field and is a key technology for structure identification. NMR is furthermore a quantitative technology. We recommend reading Koussiouris *et al.*[92] to learn more about the various advantages and disadvantages of each approach. However, it is important to note that no single method can cover the full range of metabolites present in a biological matrix.[92]

Compared with other omics, metabolomics requires minimal sample material and preparation, and is very cost-effective. In terms of computational analysis, supervised ML methods are predominantly used in metabolomics. The most frequently applied algorithm is the partial least square regression (PLSR),[93] which is usually followed by other classical ML methods, such as logistic regression (LR), random forest (RF) or SVM.[94] Looking at the order of the used algorithms, one can see that linear models are preferred, such as PLSR and LR. This is due to the easy interpretability of linear models, because there is a traceable, linear relationship between input data and output data. By contrast, the interpretability of the results for RF and SVM is more difficult, because these models are not linear. Looking at the PLSR, it has an additional advantage compared with the LR. The PLSR tries to find the best relationship between the input data and the output data (i.e., to achieve the greatest covariance between input and output). ANN, which are part of deep learning (DL), are less common in metabolomics, although their performance is comparable.[94] Its low usage is because of the large amounts of data that are needed to train deep and powerful ANN. Moreover, it is only recently that freely available software has become available with which one can easily program ANN oneself.[94] Despite that, ANN offer a high flexibility in the model architecture, which could be advantageous in the future for problems in which classical ML methods are no longer sufficient, because they can approximate linear and nonlinear problems. In this way, the models can adapt to different structures of the metabolomics data.

There has been a tremendous amount of metabolomics applications implemented to study T2DM. For instance, it has been shown that the metabolism of carbohydrates, the citrate cycle, lipids and amino acids are disturbed in T2DM, with changes in prominent metabolites including glucose, pyruvate, lactate, β-hydroxybutyrate, succinate, citrate and 2-oxoglutarate. In comparison, lipidomics research has found a drastic change in lipid profiles. This includes decreased levels of lysophospholipids, phosphatidylcholines, sphingomyelins and cholesterol esters and raised levels of triacylglycerols, diacylglycerols and phosphatidylethanolamines.[7] Owing to a high connectivity and putative crosstalk between these dysregulated metabolites and their pathways, integrating metabolomics and lipidomics is necessary to fully understand the metabolic disturbances associated. Therefore, further functional analysis of important modified molecules is key to understanding the molecular mechanism in the pathogenesis of T2DM and offers a fresh perspective in early drug development.[84,95] For example, furan fatty acid metabolite 3 carboxy-4 methyl-5 propyl-2 furanpropanoic acid (CMPF) was found to be significantly upregulated in the progression to T2DM and was identified as a potential biomarker for T2DM. Mechanism studies guided by metabolomics research showed that CMPF can directly act on β-cells, by impairing mitochondrial function, reducing glucose-induced ATP accumulation and inducing oxidative stress, leading to dysregulation of key transcription factors and decreased insulin biosynthesis.[96] This integrated study helps to understand the β cell dysfunction in diabetes, and shows that antioxidant treatment could aid prevention and treatment of T2DM.

In terms of activity metabolomics in T2DM, most metabolomics-based drug discovery studies used simple hypoglycemic activity and toxicity assays.[14] The major concept of such an assay is to evaluate enzyme inhibitory potential of natural products (NPs), traditional medicines or extracts of different origins and to consequently identify functional and bioactive metabolites out of these mixtures.[97,98] Bioactivity-guided fractionated and MS^2 networking are often applied. Although the early recognition of rediscovery of known compounds or struc-

turally related molecules by molecular MS^2 networks has vastly advanced the success of metabolomics in drug discovery, physical isolation and structure identification remain as crucial steps.[99,100].



Concluding remarks: Need for combined comprehensive techniques

Novel regulators between metabolic pathways, which can explain the fine-tuning between different physiological states and different cell types relevant for T2DM and its comorbidities, need to be identified. A major issue in discovering phenotype differences and potential drug targets in complex diseases such as T2DM is the availability of robust *in vitro* assays suitable for HTP. We have presented such HTP techniques able to decipher phenotypes on two orthogonal dimensions: morphological phenotypes and molecular phenotypes. We have shown that the currently druggable targets give distinct readouts in HCS even when using a standard cell line. This makes the technique suitable for screens for compounds having a known MOA (like in repurposing studies). Beyond this, combining the information obtained from HCS and metabolomics or lipidomics, and correlating it with previous findings, promises the possibility to discover new MOAs. For such, we suggest to incorporate a novel detection method for unknown MOAs in HCS and to combine it with metabolomics or lipidomics readouts obtained for the treated cells, which gives molecular details about metabolic pathways and metabolite classes altered upon the treatment. If the obtained molecular phenotype is intertwined in a disease-related context, one could go further to elucidate the chemical composition of the extracts that has been used for the screening or for structure elucidation in case of isolated drug candidates (Fig. 3). To achieve the former, we suggest to use differential analysis using LC-MS^2, NMR or UHR-MS. The differential analysis enables a fast concentration on compounds exclusively present in the bioactive sample and following a structural hypothesis, detection of putative rediscoveries and therefore an avenue for fast sample prioritization. The presented concept can be understood as an extended activity metabolomics, which greatly benefit from the addition of HCS.

Another perspective is to generate a disease-relevant phenotype *in vitro*. To this end, we have provided an overview of cell lines currently used in T2DM research. In addition, we believe that, with the increasing availability of patient cells through biobanking and induced pluripotent stem cell (iPSC) models, an excellent opportunity is developing for cell-based drug discovery and for studying differences in drug-induced cellular phenotypes (patient stratification, personalized medicine). DL algorithms here are particularly promising to accomplish the downstream data analysis. A fascinating paper by Schiff *et al.* has recently been published using DL combined with HCS to identify phenotype variations in Parkinson's disease.[101] The available toolbox to induce disease-related phenotypes or proceed targeted validation furthermore includes combinations and applications of genetic tools like RNA interference (RNAi) or CRISPR-Cas (clustered regularly interspaced short palindromic repeats associated proteins), to silence gene expression to determine effects on enzyme activity and metabolite production. Similarly, antimetabolites, antibiotics and immunomodulators can all be used to interfere with metabolic pathways. Bioactive lipids and their regulatory role are furthermore of high interest.

Robust HTP techniques are required for all concepts presented above. To be successful, cross-omics integration using sophisticated AI tools needs to be developed and benchmarked. Concepts about how to do so already exist for other omics integrations.[102,103] For example, combining metabolomics with other omics techniques, particularly metagenomics, has revealed the important role of the gastrointestinal microbiome, plasma dyslipidemia and inflammation in the progression and prevention of T2DM. In the past, HCS has been successfully combined with deep chemical profiling of the extract to accelerate the discovery of active compounds from complex mixtures.[8,63,104] In particular, extreme environments and ecosystems hide specific chemistry that makes them ideal natural chemical libraries for the search of novel bioactive compounds.[63] Combined orthogonal and high-resolution screening provides comprehensive access to phenotype modulations in different cell types, linking a phenotype directly to a MOA, to a fingerprint of the molecular inventory of the treated cell populations or organoids, and to knowledge of the molecular composition of the chemical compounds or extracts. At a later stage, targeted screening should be used to verify and validate the results.

Another interesting aspect worth exploring is the study of phenotypes resulting from the simultaneous modulation of several of the presented drug targets. This could be done in standard cells, as outlined above, and later in disease models or even orga-

FIGURE 3

Integrated concept for in-depth characterization of phenotypic modulations in disease models or upon treatment with single-compound complex extracts of various origins by combing HCS, metabolomics and molecular screens via artificial intelligence. The figure shows the concept of advanced phenotyping. Different extracts of interest are given in HTP to two parallel or marginally sequential phenotyping methods (HCS, metabolomics or lipidomics). First, the HCS gives a phenotypic response of the cells to the treatment with the extract (including information about toxicity, activity, MOA, SAR, subcellular target). This can be compared with prior built databases containing so-called cytological profiles of known drugs and bioactives. Additionally, a novel detection algorithm might be used to detect yet unknown MOAs. Because there are a large number of cellular responses which might be of interest, metabolomics or lipidomics is used as a secondary readout. Metabolomics reflects the response of the cells on the molecular level (MOA, metabolic network and pathways, subcellular target). The obtained 'characteristic' molecular fingerprints and also the altered metabolic pathways and classes of modified compounds can give information about the relevance of the phenotype in connection to T2DM. If a positive evaluation is also obtained in this case, we propose a differential analysis of the used extracts or fractions thereof afterwards. Through such a comparative procedure, peaks can be quickly identified that are exclusive to the sample showing the corresponding bioactivity. Depending on the method used, these peaks can be quickly connected to structural ideas for the relevant peak (in LC-MS2 via, for example, retention time and MS2 based networks, in UHR MS via accurate mass, elemental composition, isotopic fine-structure and Kendrick analogous networks, among others). This leads to a follow-up (prioritization and purification) or to discards (e.g., a rediscovery owing to similarities in structure to known compounds is present). Subsequently, the assay is validated with the purified compound or fractionated sample prior target deconvolution. A final structure elucidation needs the validation with orthogonal analytical techniques. The presented concept is acceptable to standard cell lines and disease models. Abbreviations: HTP, high-throughput; UHR, ultrahigh-resolution; MOA, mechanisms of action; AI, artificial intelligence.

noids. AI models that account for high dimensionality and having access to combinatorial effects are needed for such purposes. This is particularly important for elucidating pleiotropic effects and exploring compounds that act on multiple targets and thus have synergistic effects, such as dual- or triple-action drug candidates.

Conflicts of Interest

The authors declare that they do not have any conflicts of interest related to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.drudis.2022.07.016.

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