

Medicinal Chemistry, Metabolic Profiling and Drug Target Discovery: A Role for Metabolic Profiling in Reverse Pharmacology and Chemical Genetics

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Abstract: Comprehensive analysis of the metabolome can contribute to mechanism of action studies for small molecules discovered in phenotypic screens. Examples are presented in this overview of the rapidly developing field of “metabolic profiling.” These examples include the use of NMR in gene function analysis, GC-based studies on the identification of metabolic pathways affected by PPAR- γ agonists, applications of Fourier-transform MS and the use of stable isotope-based metabolic profiling (SIDMAP) to investigate metabolic adaptive changes induced by effective anticancer agents.

Keywords: Chemical genetics, reverse pharmacology, metabolic profiling, metabolic fingerprinting, metabonomics, metabolomics, SIDMAP.

INTRODUCTION

In the post-genomic era it remains true that the goal of the pharmaceutical industry is not simply to find novel drug targets but to find small molecule compounds that modulate their activity. A corollary, of course, is that small molecules can also be exploited to discover novel targets. This approach is sometimes referred to as ‘reverse pharmacology’ or ‘chemical genetics.’ There are now numerous examples where small molecules have been utilized to isolate and identify new drug targets and dissect signal transduction pathways [1-7]. This has often been true where traditional molecular genetic approaches have proven of limited applicability or value. Much of the research in this area has been extensively reviewed. Prominent examples of ‘tool’ or ‘probe’ compounds typically cited (Fig. 1) include fumagillin (**1**) [8, 9], trapoxin (**2**) [10-12], cyclosporin (**3**) [1, 13-16], FK506 (**4**) [1, 13-16] and rapamycin (**5**) [17-19]. Fumagillin (**1**) was implicated in the discovery of methionine aminopeptidase-2 [8, 9]. Trapoxin (**2**) was the basis of the discovery of histone deacetylase [10-12], a finding that has now facilitated investigations on the interactions between chromatin disassembly and transcriptional regulation. The immunosuppressants, cyclosporin (**3**) and FK506 (**4**), are well-studied inhibitors of the T-cell receptor that act through inhibition of calcineurin [1, 13-16]. Rapamycin (**5**) has been used extensively as a tool compound to dissect nutrient signaling pathways in yeast [17-19].

Many of the compounds that have found value as “probes” in identifying new targets or in dissecting signal transduction pathways have been complex natural products. Their discovery has generally been serendipitous and the *ad*

hoc nature of this approach is simply not commensurate with the high-throughput screening demands of today’s pharmaceutical industry. There have now been many attempts to synthesize diversity-oriented libraries of natural product-like compounds that can be utilized in high-throughput phenotypic screening [20-23]. Examples of complex natural product-like compounds from these libraries (Fig. 2) include uretupamine (**6**) [7], which activates a glucose-sensitive transcriptional pathway and has remarkable specificity towards the yeast protein ure2p, and secramine, (**7**) [24] which inhibits protein trafficking from the Golgi apparatus.

Many simpler compounds (at least in terms of fewer stereogenic centers, synthetic accessibility and lower average masses) that could be described as ‘drug-like’ have shown value in this discipline (Fig. 3). In a screen for mitosis-specific inhibitors that do not interact directly with microtubules, monastrol (**8**) [2] was shown to be a selective inhibitor of the kinesin-related motor protein, eg5. Compound A3 (**9**) [25] was identified as an inhibitor of the yeast transcriptional repressor, sir2p. Furthermore, many simple compounds have demonstrated the ability to produce phenotypes that are similar to those caused by genetic mutations [26]. Thus, for example, compounds 31J6 (**10**), 31N3 (**11**) and 32N5 (**12**) induce phenotypic changes in developing zebrafish that are highly analogous to the *breakdance*, *keinstein* and *atlantis* mutants, respectively [26].

Obstacles to a systematic reverse pharmacology or chemical genetic approach continue to be overcome through advances in diversity-oriented library design and developments in transcriptional profiling, reporter gene assays, cytoblots and automated microscopy. It is thus possible, at least in principle, to design a high-throughput chemical genetic screen for any biological process and identify compounds that can further dissect these processes

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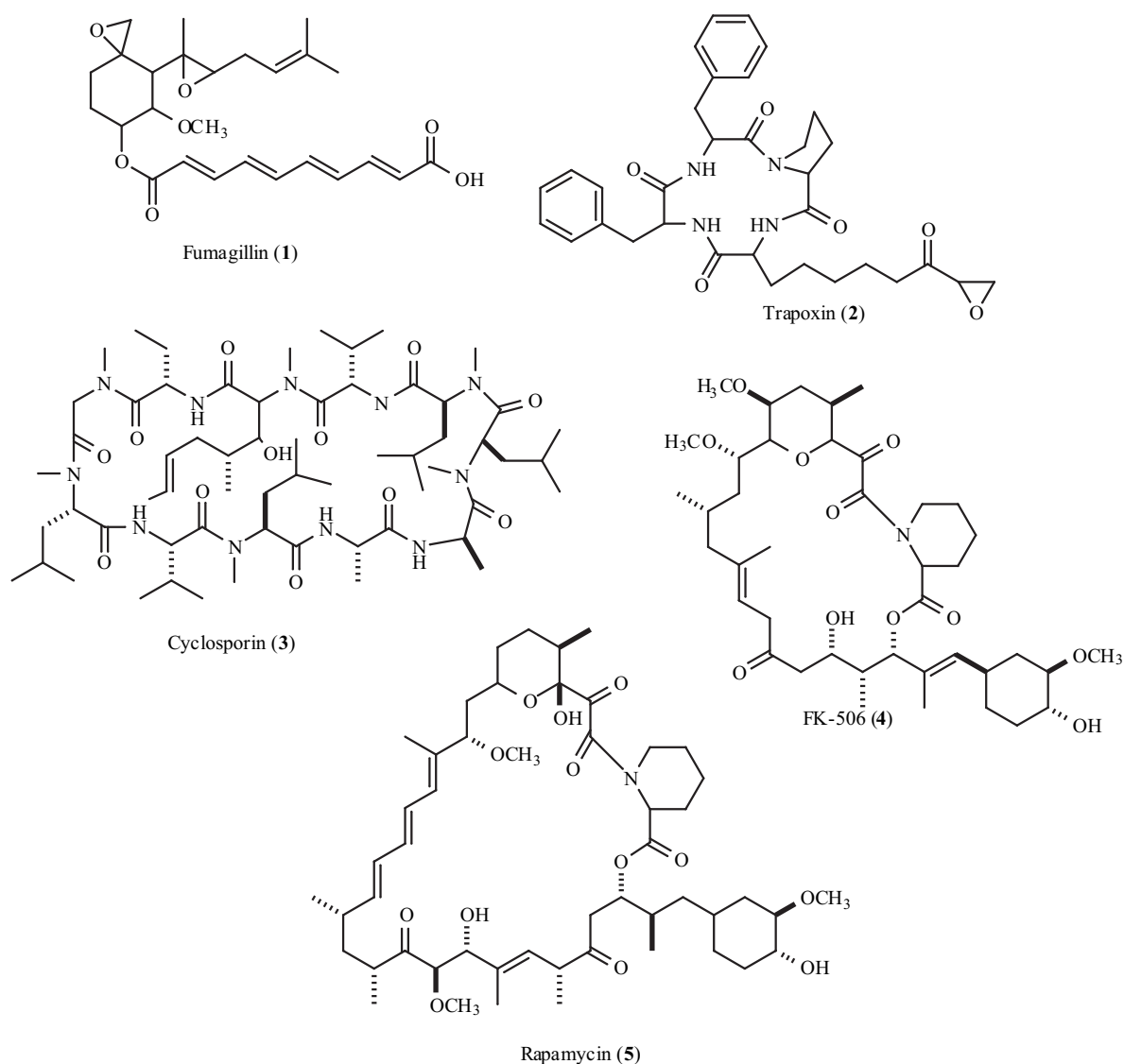


Fig. (1). Prominent examples of natural products utilized in chemical genetics studies.

[4]. In other words, the ability to systematically utilize synthetic compounds in target discovery and gene function analysis is now a reality.

Transcriptional profiling is increasingly popular in gene function analyses and in assessing the mechanism of action of “tool” compounds. Several studies have demonstrated its value in finding new disease-associated targets and as a diagnostic tool. There are, however, limitations in applying mRNA (or even protein) profiling as a general tool for reverse pharmacology approaches. The paradigm of linear control from gene expression to transcription and translation to metabolic status has been challenged by a case study on the regulation of glycolysis [27]. Low correlations between mRNA and protein abundances [28], fluctuations in RNA or protein turnover rates, and the complexity of protein interaction networks also imply further limitations.

Metabolic adaptive changes can be regarded as the end-point of all regulatory steps that respond to external perturbations, including the administration of biologically active synthetic compounds. Since even small differences in metabolic flux rates can result in pronounced changes in

metabolite abundances [29–31], analytical technologies devoted to measuring changes in metabolic status can be powerful in assessing the effects of “tool” compounds and drugs. Although the mechanism of action of synthetic compounds or even gene functions are unlikely to be pinned down definitively by metabolic profiles alone, the concentration changes observed on even minor changes in metabolic fluxes can be exploited, as will be discussed later, in screening and in comparative strategies.

Finally, both transcriptomics and proteomics are associated with high cost, limiting both the number of time-points evaluated after exposure to a given compound and the number of analyzed biological replicates. At this point, metabolomic assessments, which are considerably less expensive than transcriptomics or proteomics, may be considered.

WHAT IS METABOLIC PROFILING?

Metabolic profiling [32] involves the acquisition of metabolome data sets of sufficient spectral and/or

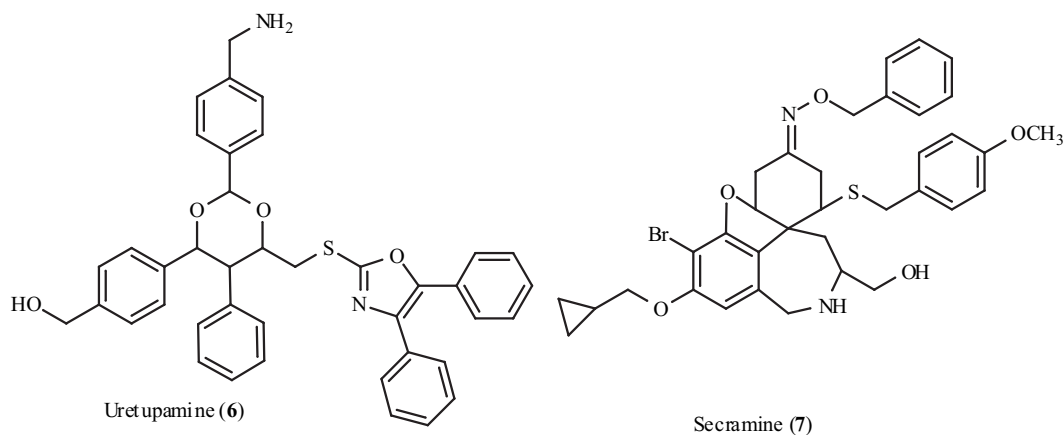


Fig. (2). Examples of natural product-like compounds synthesized in delivery libraries and utilized in chemical genetics studies.

chromatographic richness and resolution for multivariate statistical analyses and for metabolite identification and quantitation. Major strands of this discipline, as discussed below, include metabolic fingerprinting, metabonomics and metabolomics. Whilst *metabolic fingerprinting* and *metabonomics* assume it is not necessary to determine levels of all individual metabolites for classification or response readouts, *metabolomics* absolutely requires the identification and quantitation of as broad a class of metabolites as possible. Terminologies related to metabolic profiling are still developing but there appears to be consensus around the definitions provided by Fiehn [33]. Of some minor scholastic interest in regarding "metabolomics" as an extremely pertinent term for the comprehensive study of cellular and systemic metabolic changes induced by chemical, genetic or environmental factors is the etymological origin of metabolism; 'metabol' is the ancient Greek for change, 'metabolikos' means changeable.

METABOLIC FINGERPRINTING AND METABONOMICS

According to Metabolic Control Analysis [29-31], gene deletion will inevitably lead to changes in the concentration of small molecule metabolites even when phenotypic changes are negligible. Exploiting this principle, a yeast

metabolic fingerprinting method for determining gene function, particularly for genes whose deletion results in a "silent" phenotype, was recently introduced [34]. In a proof-of-principle experiment, ^1H nuclear magnetic resonance (NMR) analysis was used to measure the concentration of metabolites from different strains of yeasts including those with deletions of genes coding for enzymes involved in glycolysis and respiration. Multivariate analyses of recorded NMR spectra allowed differential clustering of mutants with different gene deletions and co-clustering of mutants with related gene deletions. Specifically, in this experiment two strains with knockouts of different phosphofructokinase genes (*pfkΔ26* and *pfkΔ27*) had similar metabolome patterns whereas strains with deletions associated with the respiratory chain had altered metabolome patterns that did not cluster with the phosphofructokinase-deficient strains (Fig. 4).

These findings imply that a stable database of metabolome patterns associated with known single-gene defects can facilitate the use of pattern recognition-based metabolic profiling to identify the function of unknown gene deletant. Other measurement technologies such as Fourier-transform infra-red spectroscopy (FT-IR) [35, 36] or mass spectrometry (MS) [37-41] have demonstrated utility in discriminating between closely related microbial species and have now been utilized in metabolome studies. Principal

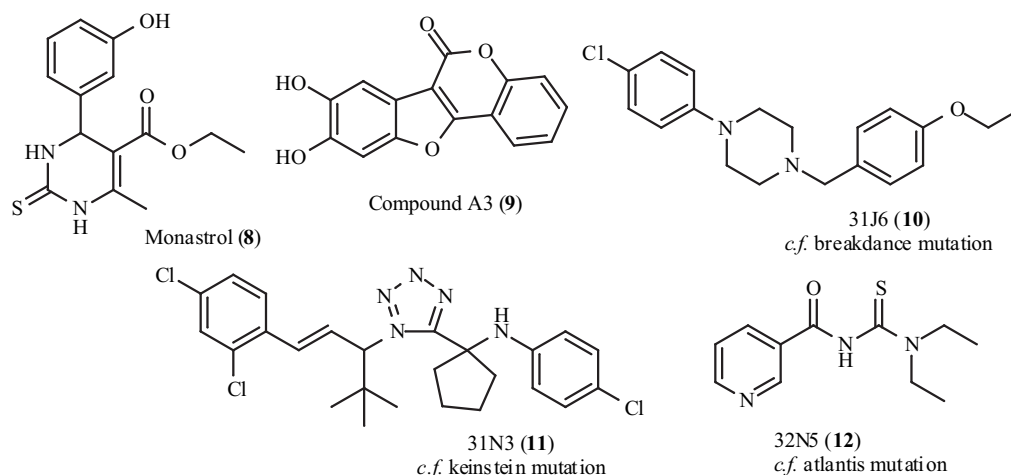


Fig. (3). Examples of "simple" compounds utilized in chemical genetics studies.

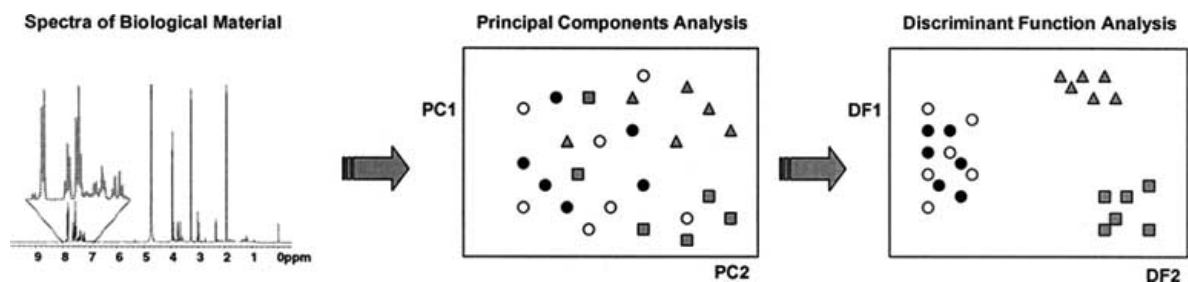


Fig. (4). The principle behind metabolic fingerprinting and metabonomics is to record spectra (in this instance an NMR spectra is presented but FT-IR, Raman and mass spectra can also be used) and reduce the dimensionality of the data through PCA to allow ready visualization of similar and dissimilar spectra. Spectra are typically normalized on both axes to allow quantitative comparisons. Selected spectral regions can be removed if desired *e.g.* such as the aromatic region of the NMR spectra in the yeast metabolic fingerprinting method (see text). Discriminant Function Analysis utilizes PC projections and “a priori” information on instrument replicates to minimize within-group variance thus maximizing between-group variance. In the yeast metabolome example presented in the text the circles would represent two mutants (six instrument replicates) with similar deletions (*e.g.* *pfkA26* and *pfkA27*) while the square and triangle would represent unrelated strains. Other routinely used chemometric options include soft independent modeling of classification analogy, partial least squares (PLS) analysis and PLS discriminant analysis. The goal, ultimately, is to determine which spectra (of gene deletants or compound treated organisms) are most similar and thereby establish which gene deletions or compound administrations yield similar or different phenotypes.

components and discriminant function analyses, as alluded to in the accompanying text for (Fig. 4), are far from the only chemometric options available, and ‘supervised’ machine learning approaches, such as neural networks or evolutionary computation, are increasingly popular [42, 43].

The fingerprinting approach utilized in the above ‘proof-of-principle’ experiment could also be applied to mammalian systems (*e.g.* as part of mutagen-driven studies on embryonic stem cells [44]) and, more pertinently to this discussion, to chemical modifiers of gene function such as biologically active small molecules. Thus, in principle, compounds identified from a phenotypic screen of mammalian biology can be rapidly assessed for similarity or differences in effecting metabolic changes. *Such low-cost metabolomic assessments could also serve as a filter or prioritization tool for expensive transcriptomics or proteomic analyses.* Significantly, the low cost of metabolic profiling allows time-series profiling. Such profiles record metabolic changes over time offering a clearer understanding of the overall effect of an administered compound on a biological system. This can optimize selection of the most functionally relevant time intervals for proteomic and transcriptomic analyses.

Importantly, such approaches as exemplified in (Fig. 4), utilize technology readily available to the pharmaceutical industry and, as such, this use of metabolic screening in mechanism-of-action studies is easily and immediately implementable. An infrastructure through COMET (Consortium on Metabonomics in Toxicology and which comprises six major pharmaceutical companies) is essentially already available for NMR based approaches [45-47]. COMET have focused primarily on screening of urine from drug-treated animals in order to associate organ toxicity of given compounds with specific spectral patterns. However the success of metabonomics in i) classifying compounds according to their toxicological impact and ii) in discriminating different strains of mice including transgenic systems [48] clearly implies that wider pharmacological applications, including reverse pharmacology or chemical genetics, are feasible.

METABOLOMIC STRATEGIES

A major advantage of the fingerprinting approach and of metabonomics is their amenability to automation and the fact that they do not require explicit identification or quantitation of specifically targeted classes of metabolites. However, several metabolic profiling approaches are focused on the identification and quantitation of as many metabolites as possible or are devoted to assessing *fluxes* through targeted pathways. Such methods, as will now be discussed, include gas chromatographic (GC)-time-of-flight (TOF)-MS, GC-flame ionization detection (FID), Fourier-transform-MS and stable isotope label based metabolic profiling (SIDMAP).

One of the earliest reported examples of reverse pharmacology using metabonomics involved the use of direct chemical ionization MS to investigate the effect of sterol biosynthesis inhibitors, such as lomebazole, on *Candida albicans* metabolic profiles [49]. In both *C. albicans* forms (hyphal and yeast-like) significant increases in the abundance of dehydrolanosterol, lanosterol and 24-demethylene-24, 25-dihydrolanosterol were recorded. These results pointed to inhibition of the C-14 demethylation step in ergosterol biosynthesis and demonstrated that lomebazole selectively inhibits cytochrome P-450 C-14 lanosterol α -demethylase. This pioneering study proved that metabolic profiling could be an effective way to investigate mechanisms of drug action.

In the 15 years or so since this work there have, of course, been major developments in MS technology and in GC peak deconvolution algorithms [50]. GC-TOF-MS is considered by some to be the “gold standard” [33, 51] for metabolomic research and it has now been increasingly applied in plant metabolism studies devoted to gene function analyses [52]. Preliminary applications in investigations of metabolic differences in transgenic animal models have also been reported [53] and it is only a matter of time before its wider application to studies on the mechanism of action of compounds discovered in phenotypic screens.

Evidence of the value of GC-based metabolomic methods in assessing the effects of synthetic compounds has recently been reported. Utilizing GC-FID, Watkins [54, 55] has reported that Lipomics Technologies (www.lipomics.com) can rigorously quantitate over 500 lipids from biological samples and present this information in a visualization package analogous to gene transcript array representations. Their lipomic data can also be processed by *in silico* algorithms that use quantitative metabolome information to predict changes in *in vivo* enzyme activities. As a recent example of their approach [54, 55], we can consider lipomic profiling applied to a study of unusual phenotypic effects induced by anti-diabetes drugs; in this instance, the metabolic actions of rosiglitazone, a PPAR- γ agonist [56] and CL, 316, 243, a β -3 adrenergic agonist [57]. On administration to mice susceptible to hepatic steatosis and type 2 diabetes these two drugs show similar reductions in serum cholesterol, glucose and triglycerides. However, rosiglitazone effected a moderate increase in body-weight whereas CL, 316, 243 effected a significant decrease. Metabolic profiling revealed clear quantitative differences between the plasma and hepatic lipid composition between mice treated with the rosiglitazone versus those treated with CL, 316, 242. Specifically, metabolites related to *de novo* fatty acid synthesis were increased on rosiglitazone treatment but decreased by treatment with CL, 316, 243. The steatosis-susceptible mouse model used in this study had an intrinsic defect in the pathway for synthesizing phosphatidylcholine for VLDL assembly and it appears that rosiglitazone inhibited compensatory pathways. Clearly this approach could be applied to evaluating the mechanism-of-action of compounds discovered in phenotypic screens in animal and cell systems. This study also emphasized that, because many of the effects of these drugs on tissue metabolism were reflected in plasma composition,

“metabolomics has excellent potential for developing clinical assessments of metabolic response to drug therapy” and that metabolic profiling has implications well beyond reverse pharmacology experiments.

The high resolving power of Fourier transform-MS [58] has also been applied to comprehensive metabolome analyses. For example, Esperion Therapeutics (www.esperion.com) investigated metabolic changes in rat hepatocytes treated with lovastatin [59]. The purpose of this study was to quantify concentration-specific cellular responses to treatment by lovastatin on as many low molecular weight metabolites as possible. Over 700 peaks corresponding to individual metabolites were observed in cell lysates and supernatants from lovastatin (and control) treated samples. Of these, 36 exhibited a positive correlation with respect to their relative abundance and the concentration of administered lovastatin, whereas 42 exhibited a negative correlation. Steps were subsequently taken to identify these metabolite peaks using Esperion Therapeutics' proprietary data handling packages. Findings included a reduction in squalene and propionic acid, which is consistent with the mechanism of action of lovastatin. A reduction in saturated fatty acids, (lauric acid, myristic acid, palmitic acid, and stearic acid) indicated that flux through fatty acid synthase was reduced. There was also a concomitant decrease in unsaturated fatty acids such as myristoleic acid, palmitoleic acid and oleic acid. Also observed were reductions in the relative abundance of hexadecenal, sphingosine and sphinganine implying perturbation of the sphingoglycolipid biosynthesis pathway (Fig. 5), either by direct action on enzymes involved in this pathway or modulation of an allosteric regulator. In essence, multiple pathways affected by drug treatment could be detected by FT-MS metabolomics technology *without any prior knowledge* on which pathways would be susceptible to compound-induced

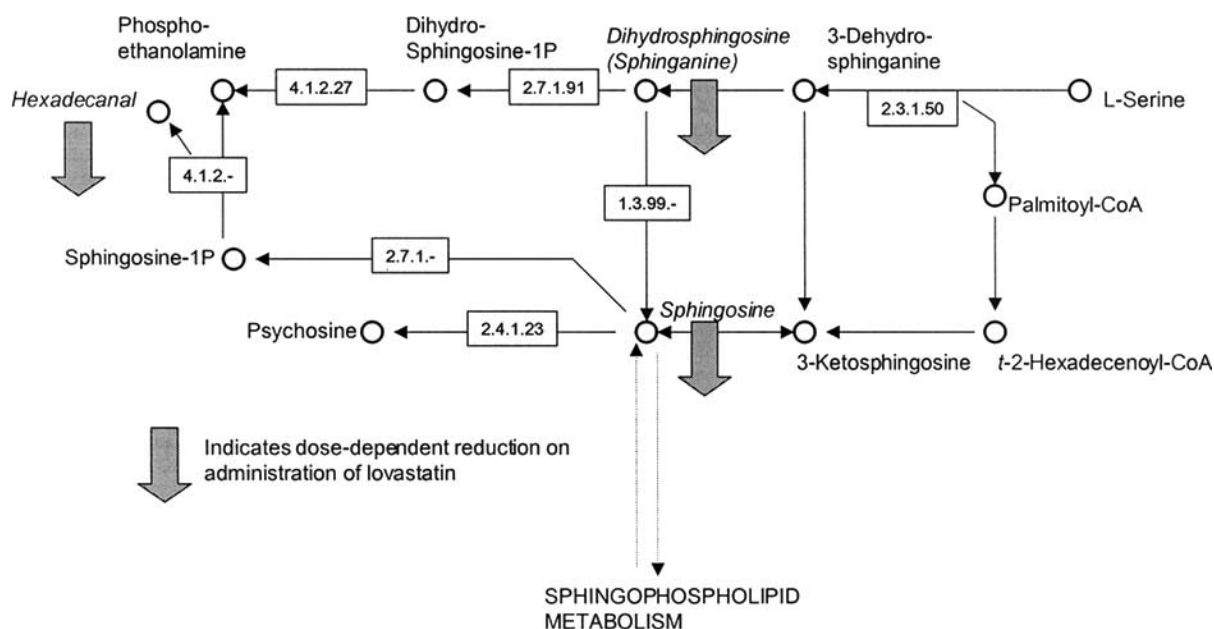


Fig. (5). Reduction in the highlighted metabolites, indicating a perturbation in the sphingoglycolipid biosynthetic pathway, as well as reduction in identified saturated and unsaturated fatty acids were recorded in a *single* FT-MS measurement of lovastatin-treated rat hepatocytes. This illustrates the value of metabolic profiling in screening the effect of compounds on multiple pathways despite no prior knowledge of pathways susceptible to perturbation. Additionally, [54] “comprehensive analyses of the metabolome can assess metabolic response to a therapy with much greater accuracy and power than biomarker approaches.”

Table 1. Adaptive Changes Identified by Stable Isotope Based Metabolic Profiling

Treatment	Target Pathways	Metabolic Profile	Phenotype
Gleevec (ST1571) (Bcr/Abl tyrosine kinase inhibitor)	Glucose intake, pentose cycle, fatty acid synthesis	Decreased glucose intake and carbon flow through the oxidative pentose cycle; decreased fatty acid synthesis; inhibitor of hexokinase and G6PD	Decreased cell proliferation, apoptosis
Genistein	Pentose cycle, fatty acid synthesis	Decreased glucose intake and carbon flow through the oxidative pentose cycle; decreased fatty acid synthesis	apoptosis
Avemar	Pentose cycle, fatty acid synthesis	Decreased glucose intake and carbon flow through the oxidative pentose cycle; increased fatty acid synthesis	Decreased cell proliferation, apoptosis
Dehydroepiandrosterone sulfate (G6PD inhibitor)	Pentose cycle	Decreased oxidative pentose cycle flux	G1 cell cycle arrest
Oxythiamine (transketolase inhibitor)	Pentose cycle	Decreased non-oxidative pentose cycle flux	G1 cell cycle arrest

changes. The information generated by FT-MS can easily be integrated with an existing reference databases of metabolic pathways [60] and with transcriptomic and proteomic analyses.

Another company focusing on the use of FT-MS in metabolomic studies is Phenomenome Discoveries, Inc. (www.phenomenome.com) Their bioinformatics package includes a metabolome array analogous to gene transcript arrays [61]. They have now reported on a metabolomic investigation of two histone deacetylase inhibitors, butyrate and trichostatin A [12]. Despite the same reported mode of action, differential “expression” of metabolic pathways was highlighted by FT-MS analyses when these compounds were separately administered to HT29 human colon adenocarcinoma cells. Cluster analyses (PCA) of the FT mass spectra clearly distinguished the two different compound treatments at 24 hours.

While the above methods are based on exploiting changes in metabolite concentrations, other powerful technologies include those based on assessing changes in metabolic fluxes on compound administration. Stable isotope-based metabolic profiling (SIDMAP) methods in which labeled precursor molecules such as [1, 2-¹³C₂]glucose are applied to cell culture systems and the ¹³C distribution patterns assessed by GC [62-64] (or even NMR [e.g. 65]) technologies are proving to be valuable. This approach has been used to investigate metabolic adaptive changes induced by novel and effective anticancer agents. Transformed cells exhibit unique anabolic characteristics, which includes increased and preferential utilization of glucose through the non-oxidative steps of the pentose cycle for nucleic acid synthesis, but limited *de novo* fatty acid synthesis and TCA cycle glucose oxidation. This primarily non-oxidative anabolic profile reflects an undifferentiated highly proliferative aneuploid cell phenotype and serves as a reliable metabolic biomarker to determine cell proliferation rate and the level of cell transformation/differentiation in response to drug treatment. Drugs effective in particular cancers exert their anti-proliferative effects by inducing significant reversions of a few specific non-oxidative anabolic pathways. This is summarized in Table 1.

Thus compounds discovered in phenotype screens related to oncogenesis (e.g. apoptosis screens, G1 arrest) could be prioritized by metabolic profiles showing desired metabolic adaptations for further more extensive (and expensive) studies.

INFORMATICS IMPLICATIONS

Unlike metabolic fingerprinting or metabonomic strategies, which do not require explicit quantitation of metabolites (and have relatively straightforward informatics requirements), metabolomics requires that the recorded metabolome data sets be processed algorithmically to yield a list of metabolites (known or unknown) along with a measure of their absolute or relative concentrations. As with metabolic fingerprints, such data sets can be subjected to chemometric analyses, including the use of supervised machine learning [66]. A recent demonstration of the use of pairwise correlations to analyze metabolome data sets represents a stimulating approach to establishing a relationship between metabolome data sets and pathway analysis [67].

Reference biochemical databases are also important to metabolomic research and many such databases (usually devoted to single species) are in the public domain [60, 68]. Such databases catalog the known biochemical compounds, reactions, enzyme activities, proteins and genes for each organism and are required to provide biological and chemical context to measurements made by metabolomic technologies.

CONCLUDING REMARKS

Metabolic profiling represents a logical development in the paradigm of genomics, transcriptomics and proteomics. It may prove to be the most practically relevant and robust discipline of all, particularly when applied to reverse pharmacology or chemical genetic experiments. Even the relatively simple fingerprinting and metabonomic strategies are proving to have a substantial impact on several areas of drug discovery and development. The continuing development of new approaches to generate interrogate metabolome data sets implies that metabolic profiling will

continue to extend its value in mechanism of action (and other) studies.

It is suggested herein that reverse pharmacology or chemical genetics, the use of medicinal chemistry to discover drug targets and dissect signal transduction pathways, will benefit greatly from the information generated through metabolic profiling.

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REFERENCES

- [1] Hung, T.; Jamison, T.F.; Schreiber, S.L. *Chem. Biol.* **1996**, *3*, 623.
- [2] Crews, C.M. *Chem. Biol.* **1996**, *3*, 961.
- [3] Crews, C.M.; Splittgerber, U. *Trends Biochem. Sci.* **1999**, *24*, 317.
- [4] Haggarty, S.J.; Mayer, T.U.; Miyamoto, D.T.; Fathi, R.; King, R.W.; Mitchison, T.J.; Schreiber, S.L. *Chem. Biol.* **2000**, *7*, 275.
- [5] Stockwell, B.R. *Trends Biotech.* **2000**, *18*, 449.
- [6] Crews, C.M.; Mohan, R. *Curr. Opin. Chem. Biol.* **2000**, *4*, 47.
- [7] Kuruvilla, F.G.; Shamji, A.F.; Sternson, S.M.; Hergenrother, P.J.; Schreiber, S.L. *Nature* **2002**, *416*, 653.
- [8] Sin, N.; Meng, L.; Wang, M.Q.W.; Wen, J.J.; Bornmann, W.G.; Crews, C.M. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 6099.
- [9] Liu, S.; Widom, J.; Kemp, C.W.; Crews, C.M.; Clardy, J. *Science* **1998**, *282*, 1324.
- [10] Kijima, M.; Yoshida, M.; Sugita, K.; Horinouchi, S.; Beppu, T. *J. Biol. Chem.*, **1993**, *268*, 22429.
- [11] Taunton, J.; Collins, J.L.; Schreiber, S.L. *J. Am. Chem. Soc.* **1996**, *118*, 10412.
- [12] Grozinger, C.M.; Schreiber, S.L. *Chem. Biol.* **2002**, *9*, 3.
- [13] Schreiber, S.L. *Science* **1991**, *251*, 283.
- [14] Liu, J.; Farmer, J.D.; Lane, W.S.; Friedman, J.; Weissman, I.; Schreiber, S.L. *Cell* **1991**, *66*, 807.
- [15] Schreiber, S.L. *Cell* **1992**, *70*, 365.
- [16] Liu, J. *Immunol. Today* **1993**, *14*, 290.
- [17] Heitman, M.; Mow, N.R.; Hall, M.N. *Science* **1991**, *253*, 283.
- [18] Hardwick, J.S.; Kuruvilla, F.G.; Tong, J.K.; Shamji, A.F.; Schreiber, S.L. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 14866.
- [19] Shamji, A.F.; Kuruvilla, F.G.; Schreiber, S.L. *Curr. Biol.* **2000**, *10*, 1574.
- [20] Tan, D.S.; Foley, M.A.; Shair, M.D.; Schreiber, S.L. *J. Am. Chem. Soc.* **1998**, *120*, 8565.
- [21] Tan, D.S.; Foley, M.A.; Stockwell, B.R.; Shair, M.D.; Schreiber, S.L. *J. Am. Chem. Soc.* **1999**, *121*, 9073.
- [22] Schreiber, S.L. *Science* **2000**, *287*, 1964.
- [23] Sternson, S.M.; Wong, J.C.; Grozinger, C.M.; Schreiber, S.L. *Org. Lett.* **2001**, *3*, 4239.
- [24] Pelish, H.E.; Feng, Y.; Peterson, J.R.; Westwood, N.J.; Tsai, A.L.; Kirschner, M.W.; Kirshausen, T.; Shair, M.D. *Abs. Pap. Am. Chem. Soc.* **2002**, *224*, 200.
- [25] Grozinger, C.M.; Chao, E.D.; Blackwell, H.E.; Moazed, D.; Schreiber, S.L. *J. Biol. Chem.* **2001**, *276*, 38837.
- [26] Peterson, R.T.; Link, B.A.; Dowling, J.E.; Schreiber, S.L. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 12965.
- [27] ter Kuile, B.H.; Westerhoff, H.V. *FEBS Lett.* **2001**, *500*, 169.
- [28] Gygi, S.P.; Rochon, Y.; Franza, B.R.; Aebersold, R. *Mol. Cell Biol.* **1999**, *19*, 1720.
- [29] Kacser, H.; Burns, J.A. *Symp. Soc. Exp. Biol.* **1973**, *27*, 65.
- [30] Fell, D.A. *Understanding the Control of Metabolism*, Portland Press: London, **1996**.
- [31] Heinrich, R.; Schuster, S. *The Regulation of Cellular Systems*, Chapman and Hall; New York, **1996**.
- [32] Harrigan, G.G.; Goodacre, R., (Eds.) *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*, Kluwer Academic Publishers; Boston, **2003**.
- [33] Fiehn, O. *Compar. Funct. Genom.* **2001**, *2*, 155.
- [34] Raamsdonk, L.M.; Teusink, B.; Broadhurst, D.; Zhang, N.S.; Hayes, A.; Walsh, M.C.; Berden, J.A.; Brindle, K.M.; Kell, D.B.; Rowland, J.J.; Westerhoff, H.V.; van Dam, K.; Oliver, S.G. *Nature Biotechnol.* **2001**, *19*, 45.
- [35] Goodacre, R.; Timmins, É.M.; Burton, R.; Kaderbhai, N.; Woodward, A.; Kell, D.B.; Rooney, P.J. *Microbiology* **1998**, *144*, 1157.
- [36] Oliver, S. G.; Winson, M. K.; Kell, D. B.; Baganz, F. *Trends Biotechnol.* **1998**, *16*, 373.
- [37] Higgs, R.E.; Zahn, J.A.; Gygi, J.D.; Hilton, M.D. *Appl. Env. Microbiol.* **2001**, *67*, 371.
- [38] Zahn, J.A.; Higgs, R.E.; Hilton, M.D. *Appl. Env. Microbiol.* **2001**, *67*, 377.
- [39] Vaidyanathan, S.; Rowland, J.J.; Kell, D.B.; Goodacre, R. (2001). *Anal. Chem.* **2001**, *73*, 4134.
- [40] Vaidyanathan, S.; Kell, D.B.; Goodacre, R. *J. Am. Soc. Mass Spectrom.* **2001**, *13*, 118.
- [41] Vaidyanathan, S.; Winder, C.L.; Wade, S.C.; Kell, D.B.; Goodacre, R. *Rapid Comm. Mass Spectrom.* **2002**, *16*, 1276.
- [42] Goodacre, R.; Kell, D.B. In *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*; Harrigan, G.G.; Goodacre, R. Eds., Kluwer Academic Publishers; Boston, **2003**; pp. 239-256.
- [43] Kell, D.B.; Darby, R.M.; Draper, J. *Plant Physiol.* **2001**, *126*, 943.
- [44] Balling, R. *Annu. Rev. Genomics Hum. Genet.* **2001**, *2*, 363.
- [45] Nicholson, J.K.; Connelly, J.; Lindon, J.C.; Holmes, E. *Nature Rev. Drug Discovery* **2002**, *1*, 153.
- [46] Breau, A.P.; Cantor, G.H. In *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*; Harrigan, G.G.; Goodacre, R. Eds., Kluwer Academic Publishers; Boston, **2003**; pp.69-82.
- [47] Nicholson, J.K.; Lindon, J.C.; Holmes, E. *Xenobiotica* **1999**, *29*, 1181.
- [48] Gavaghan, C.L.; Holmes, E.; Lenz, E.; Wilson, I.D.; Nicholson, J.K. *FEBS Lett.* **2000**, *484*, 169.
- [49] Tas, A.C.; Bastiaanse H.B.; van der Greef, J.; Kerkenaar, A. *J. Anal. Appl. Pyrol.* **1989**, *14*, 309.
- [50] www.leco.com
- [51] Fiehn, O. *Plant Mol. Biol.* **2002**, *48*, 155.
- [52] Fiehn, O., Kopka, J.; Dörmann, P.; Altmann, T.; Trethewey, R.N.; Willmitzer, L. *Nature Biotechnol.* **2000**, *18*, 1157.
- [53] Fiehn O.; Spranger, J. In *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*; Harrigan, G.G.; Goodacre, R. Eds., Kluwer Academic Publishers; Boston, **2003**; pp.199-216.
- [54] Watkins, S.M.; Reifsnnyder, P.R.; Pan, H.-j.; German, J.B.; Leiter, E.H. *J. Lipid Res.* **2002**, *43*, 1809.
- [55] Watkins, S.M. Cambridge Healthtech Institute Second Annual *Metabolic Profiling: Pathways in Discovery* Conference, Research Triangle Park, North Carolina, December 2-3, **2002**.
- [56] Camp, H.S.; Li, O.; Wise, S.C.; Hong, Y.H.; Frankowski, C.L.; Shen, X.Q.; Vanbogelen, R.; Leff, T. *Diabetes* **2000**, *49*, 539.
- [57] Bloom, J.D.; Dutia, M.D.; Johnson, B.D.; Wissner, A.; Burns, M.G.; Largis, E. E.; Dolan, J.A.; Claus, T.H. *J. Med. Chem.* **1992**, *35*, 3081.
- [58] Marshall, A.G.; Hendrickson, C.L. *Int. J. Mass Spectrom.* **2002**, *215*, 59.
- [59] He, F. Cambridge Healthtech Institute Second Annual *Metabolic Profiling: Pathways in Discovery* Conference, Research Triangle Park, North Carolina, December 2-3, **2002**.
- [60] Li, X.J.; Brazhnik, O.; Kamal, A.; Guo, D.; Lee, C.; Hoops, S.; Mendes, P. In *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*; Harrigan, G.G.; Goodacre, R. Eds., Kluwer Academic Publishers; Boston, **2003**; pp. 293-210.
- [61] Goodenowe, D. In *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*; Harrigan, G.G.; Goodacre, R. Eds., Kluwer Academic Publishers; Boston, **2003**; pp. 125-139.
- [62] Boros, L.G.; Cascante, M.; Lee, W.-N.P. *Drug Discov. Today* **2002**, *7*, 364.
- [63] Boros, L.G.; Cascante, M.; Lee, W.-N.P. *Metabolic Profiling: Its Role in Biomarker Discovery and Gene Function Analysis*, Harrigan, G.G.; Goodacre, R. Eds., Kluwer Academic Publishers; Boston, **2003**; pp. 141-169.
- [64] Cascante, M.; Boros, L.G.; Comin-Anduix, B.; de Aturi, P.; Centelles, J.J.; Lee, W.-N.P. *Nature Biotechnol.* **2002**, *20*, 243.

- [65] Lu, D.; Mulder, H.; Zhao, P.; Burgess, S.C.; Jensen, M.V.; Kamzolova, S.; Newgard, C.B.; Sherry, A.D. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2708.
- [66] Taylor, J.; King, R.D.; Altmann, T.; Fiehn, O. *Bioinformatics* **2002**, *18* (Suppl 2), S241.
- [67] Kose, F.; Weckwerth, W.; Linke, T.; Fiehn, O. *Bioinformatics* **2001**, *17*, 1198.
- [68] Mendes, P. *Brief. Bioinform.* **2003**, *3*, 134.