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NMR and MS-based Stable Isotope-Resolved Metabolomics and Applications in Cancer Metabolism

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Abstract

There is considerable interest in defining metabolic reprogramming in human diseases, which is recognized as a hallmark of human cancer. Although radiotracers have a long history in specific metabolic studies, stable isotope-enriched precursors coupled with modern high resolution mass spectrometry and NMR spectroscopy have enabled systematic mapping of metabolic networks and fluxes in cells, tissues and living organisms including humans. These analytical platforms are high in information content, are complementary and cross-validating in terms of compound identification, quantification, and isotope labeling pattern analysis of a large number of metabolites simultaneously. Furthermore, new developments in chemoselective derivatization and in vivo spectroscopy enable tracking of labile/low abundance metabolites and metabolic kinetics in real-time. Here we review developments in Stable Isotope Resolved Metabolomics (SIRM) and recent applications in cancer metabolism using a wide variety of stable isotope tracers that probe both broad and specific aspects of cancer metabolism required for proliferation and survival.

Keywords

SIRM; cancer metabolism; NMR; mass spectrometry; model systems

1. Introduction

Isotope tracing approaches in metabolic studies have a long history, where a biological system is given an isotopically enriched precursor such that its metabolic transformations can be tracked by the tracer atom. Due to the much better availability as well as ease and high sensitivity of detection, radioisotopes were much preferred over stable isotopes in early tracer studies and have been instrumental in deciphering both central (e.g. the Krebs cycle) and secondary metabolic pathways [5]. However, stable isotope tracers are non-hazardous, more amenable to human studies, and can be analyzed by both NMR and mass spectrometry (MS). Radioisotopes are NMR-silent except for ^{3}H, which compromises the structural elucidation of the transformed products. Recent recognition that our understanding of cell and tissue metabolism in various human disease is grossly inadequate has driven greatly

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improved analytical techniques (especially NMR and MS) concomitant with the dramatically increased availability of various stable isotope-enriched precursors. Both advances have led to a resurgence of interest in utilizing stable isotope tracer approaches in metabolic studies. When coupling stable isotope tracers with advanced metabolomic technologies, such as Stable Isotope-Resolved Metabolomics (SIRM) [6–8], metabolic networks can now be systematically and rigorously tracked using the combined structural capabilities of NMR and MS. Figure 1 shows the pipeline of SIRM studies in different biological systems, which can be readily incorporated into a systems biochemistry workflow. Since 2000, more than 1300 articles on cancer metabolism with stable isotope tracing have been published using these search terms via Clarivate Web of Science.

In this review, we focus on recent applications of SIRM in cancer metabolism. Earlier work [9–19] and MS and NMR methodologies [20–27] have been extensively reviewed elsewhere.

2. Analytical Tools for SIRM: NMR and Mass spectrometry

The additional neutron(s) in the nucleus of a rare stable isotope enables simple detection by both NMR and mass spectrometry. For example, of the natural stable isotopes of carbon, nitrogen, and hydrogen, the rare (1.1 % abundance) ^{13}C atom is ~1 Da heavier than the abundant ^{12}C atom (98.9%), and similarly for ^{15}N (0.35%) versus ^{14}N and ^{2}H (0.02%) versus ^{1}H. However, two or more different tracer atoms such as ^{13}C, ^{15}N, and ^{2}H cannot be distinguished in the same samples with typical resolution-MS. The advances in ultra high-resolution Fourier transform mass spectrometry (UHR-FTMS) with mass-to-charge ratio (m/z) resolution to the fifth decimal place for most metabolites now make it feasible to distinguish molecules with multiple stable isotope atoms of multiple elements. This is because the additional neutron mass in for example, ^{13}C is different from that in ^{15}N or ^{2}H, so that their isotopologues (identical compounds differing by the number of tracer atoms [28]) are distinguishable by UHR-FTMS (Figure 2). This makes multiplexed Stable Isotope Resolved Metabolomics (mSIRM) experiments with the use of multiple tracer atoms readily feasible [29]

Stable isotopes such as ^{13}C can also be detected directly by NMR, whereas ^{12}C and radioactive ^{14}C are invisible to NMR. More importantly, ^{15}N and ^{13}C can be observed indirectly by the attached proton, thereby affording much higher sensitivity than direct detection [30]. As these nuclei resonate at widely different radiofrequencies (i.e. chemical shifts), mSIRM can also be achieved.

The power of NMR and MS techniques in metabolic studies lies in their versatility, complementarity and rigor in determining isotopically enriched metabolite structures and concentrations, particularly in terms of the number (isotopologues) and positions (positional isotopomers [28]) of the tracer atoms present. The two techniques are cross-validating as they analyze metabolite structures and quantify them based on entirely different physical principles. They are also complementary as NMR can more readily provide information on positional isotopomers than MS and vice versa for information on isotopologues of given metabolites. Combining both types of information with the appropriate experimental design more robustly reveal the paths of metabolic transformations and detailed metabolic fluxes

(e.g. via time course and/or flux modeling analysis). This can all be achieved in unfractionated extracts of cells, tissues, or organs, and sometimes in whole organisms.

Furthermore, an added, unique advantage of NMR is its ability to observe metabolic transformations and their rates (fluxes) in live tissues or organisms *in situ*. Traditionally, in vivo NMR mainly used ^{31}P NMR to determine the levels of ATP, inorganic phosphate, sugar phosphates and phosphocreatine as well as ATP turnover rates and intracellular pH [31, 32]. In recent years, stable isotope tracers have been used in both animal systems and humans [33–37] especially in combination with the new hyperpolarization methods (e.g. dynamic nuclear polarization or DNP) for short segments of metabolic pathways [38, 39].

3. Stable isotope tracers for metabolic studies

The most commonly used stable isotope in metabolic studies – such as in cancer metabolism research – is ^{13}C. There are numerous ^{13}C-enriched precursors commercially available, including several isotopomers of D-glucose (e.g. [U-^{13}C], [^{13}C-1,2], [^{13}C-1,6], [^{13}C-3,4]) for probing different routes of glucose metabolism, ^{13}C-octanoate [40, 41], -palmitate and -acetate for probing fatty acid metabolism and fates of intracellular ^{13}C-acetyl CoA [42, 43], ^{13}C-fructose, and ^{13}C-amino acids. It is also common to use ^{15}N-enriched amino acids either as ^{15}N only or double element labeled versions such as [U-^{13}C,^{15}N]-glutamine [44] for multiplexed SIRM studies. Such a wide variety of stable isotope-labeled sources opens a rich avenue of experimental designs for interrogating the vast and intricate metabolic networks in mammalian systems. Table 1 summarizes some commonly used, commercially available stable isotope tracers and example metabolic pathways they probe.

4. Selective detection of a class of stable isotope-enriched metabolites

Phosphorus is an important biological element, but the major stable isotope ^{31}P is close to 100% abundant. ^{31}P is a spin ½ nucleus with a gyromagnetic ratio 40% as large as that of the proton. Therefore it is a good NMR nucleus, and can be used to filter or edit a complex mixture for the detection of phosphorylated compounds, which may also be enriched with ^{13}C or ^{15}N. The simplest editing experiment is the ^{1}H{^{31}P} HSQC experiment which detects only metabolites that contain the three bond ^{31}P-O-CH coupling. These include all phosphorylated metabolites such as nucleotides, phosphosugars and intermediates of the pentose phosphate pathway [75]. This experiment greatly simplifies the ^{1}H NMR spectrum of a complex unfractionated biological extract, A wide variety of edited NMR experiments can be used to select for different biologically important nuclei particularly ^{13}C and ^{15}N. However, it may be difficult to assign metabolites reliably based on this experiment alone. HSQC-TOCSY experiments relay magnetization to additional protons to enable mapping of more of the covalent network in phosphorylated metabolites [22, 75]. If the protons are attached to ^{13}C, then their ^{13}C satellites will be observable and quantifiable in the HSQC-TOCSY spectrum, and thus provide information on the site-specific enrichment in phosphorylated metabolites [20]. Such ^{13}C and/or ^{15}N isotopomer analysis of metabolites via spectral editing decreases spectral crowding that can overwhelm low abundant metabolites, and such selection is difficult to accomplish with MS analysis. An alternative

approach to achieve selective detection of metabolites is the use of chemoselective derivatization as described below.

Systematic and reliable compound identification in crude extracts already requires significant effort, even with the combination of multidimensional NMR, tandem high-resolution MS, and extensive compound libraries. The problems are greatly exacerbated in SIRM studies where the multiple isotopomers and isotopologues of the many labeled metabolites must be identified and quantified. In fact, each isotopologue and isotopomer of a biochemical is essentially a different metabolite because they have different metabolic provenance, which are clues that their function and fate may be different. That is, the metabolic biosynthetic history is “encoded” in the NMR and MS spectra of each enriched biochemical.

This spectral encoding precludes identification by simple NMR and MS spectral matching. Moreover the presence of labeled metabolites causes greater spectral crowding, and also reduces the detectability of given metabolites by NMR and MS as each metabolite signal is split into multiple signals.

Chemoselective derivatization is a means of reducing spectral crowding while enhancing detection both by NMR (via spectral editing) and by MS. Several chemoselective probes have been reported, including cholamine for carboxylate groups [76], *N*-(2-^{15}*N*-aminooxyethyl)-*N,N*-dimethyl-1-dodecylammonium (QDA) for aldehydes and ketones [1, 77, 78], *N*-(2-^{15}*N*-iodoacetamido)-*N,N*-dimethyl-1-dodecylammonium (QDE) for sulfhydryl [4] and ethylchoroformate (ECF) for amino, hydroxyl, and carboxyl groups [29]. Each chemoselective derivatizing agent places a spectrally discernable tag on one or a few particular functional groups, which not only help metabolite identification but also quantification by NMR and MS. For example, the QDA or QDE probe contains a permanent positive charge, which facilitates ionization and thus detectability in MS (Figure 3). In addition, stable isotope-enriched versions of QDA or QDE (e.g. $^{13}CD_3$-QDA) can be prepared and reacted with metabolites as 1:1 mixture with the unlabeled probe. This greatly facilitates the identification and quantification of carbonyl or thiol containing metabolites simply by detecting a pair of adducts with an m/z difference of 4.02188 using UHR-FTMS (Figure 3).

Furthermore, this QDA probe can be modified to bear a ^{15}N-aminooxy group, which reacts with carbonyl metabolites such as pyruvate to yield a ketoxime adduct with 3-bond coupling of ^{15}N to the adjacent protons (Figure 4A). This coupling enables ^{15}N-edited HSQC detection of the adduct in both 1D and 2D experiments, which would otherwise be impractical to discern in unedited ^{1}H NMR analysis (Figure 4B–C).

Thus, chemoselective derivatization can be coupled with stable isotope enrichment and spectral editing to facilitate the analysis of low abundance metabolites whose NMR or MS signals are typically swamped by those of more abundant metabolites. It is also important to note that the derivative formation helps stabilize labile (e.g. 4-hydroxynonenal) and volatile metabolites (e.g. acetone) for NMR and MS analysis.

5. Applications in Cancer Metabolism

As noted above, metabolic reprogramming is now recognized as a hallmark of human cancer [79] and cancer metabolism studies have most frequently taken advantage of the stable isotope tracer approach. The transformation of a normal cell to a highly proliferative cancer state is associated with numerous metabolic perturbations, which are often interactively linked to changes in the transcriptional program driven by alterations of critical oncoproteins and/or tumor suppressors. During growth, the increasingly hostile tumor microenvironment can further promote metabolic reprogramming to support tumor progression [80–82]. Most solid tumors up-regulate glycolysis and convert a high fraction of the glucose consumed to lactate (i.e. the Warburg effect), which is excreted along with a proton to acidify the extracellular space. In order to support cell growth, survival, and eventual metastasis, other aspects of the metabolism must also be up-regulated, especially the synthesis of precursors for nucleic acids, lipids, complex carbohydrates and proteins, e.g. serine and glycine (for purine biosynthesis, one-carbon metabolism), aspartate (for pyrimidine biosynthesis), citrate (for lipid biosynthesis), UDP-glucose (for glycogen synthesis), non essential amino acids such as alanine, glutamate, proline for protein synthesis [83], and glutamate (for antioxidant glutathione synthesis). It is also becoming clear that the metabolism of essential amino acids, particularly the branched chain amino acids Ile, Leu and Val are important in the development of some cancers [84].

Enhanced lactic fermentation is readily determined using ^{13}C-glucose, by tracking the ^{13}C fate in cell culture and organ systems from the uptake of ^{13}C-glucose to the excretion of ^{13}C-lactate in terms of both levels and fractional enrichment as well as the fraction of glucose conversion to lactate. The excretion of ^{13}C-Ala and ^{13}C-Glu (due to exchange with extracellular cystine) are often observed in the ^{13}C-glucose tracer studies of cancer cells and tissues, which informs the status of Ala metabolism via the alanine aminotransferase (ALT) activity and the demand for glutathione (cysteine being a precursor). The fate of individual ^{13}C atoms of glucose into intracellular metabolites can also be traced, to map both central catabolic and anabolic pathways that are keys to supporting cancer cell proliferation and survival. These include, but not limited to, the pentose phosphate pathway (PPP), the Krebs cycle, Ser-Gly-one carbon metabolism, and the synthesis of glutathione, non-essential amino acids, purine/pyrimidine nucleotides, nucleotide sugars (e.g. UDP N-acetylglucosamine or UDP-GlcNAc), glycogen, and lipids. Moreover, the incorporation of newly synthesized (i.e. labeled) non-essential amino acids into proteins and nucleotides into RNA can be determined following proper hydrolysis of the macromolecules [29, 85]; such “metabolomics of biomacromolecules” is in its infancy, but these large sinks and sources of metabolites are critical to detect and quantify.

The PPP is important both for generating cytoplasmic NADPH (required for anabolic and detoxification metabolism) and ribose for nucleotide synthesis. Thus, proliferating cancer cells have a strong requirement for the PPP activity. However, it is often unclear the extent of which the PPP contributes to NADPH production via the oxidative branch in cancer cells. It is feasible to discriminate the activity of the oxidative from non-oxidative branches using specific ^{13}C isotopomers of glucose, such as [^{13}C-1,2]-glucose analyzed via MS analysis [86] or a mixture of [^{13}C-1]- and [^{13}C-2]-glucose by NMR analysis [41].

Although accelerated lactic fermentation is a common adaptation in cancer cells, there are numerous other adaptations that generate metabolic energy and anabolic precursors that are equally important. It was long conjectured that mitochondria are non-functional in cancers. However, tracer studies with ^{13}C-glucose, ^{13}C-Gln and ^{13}C-fatty acids clearly demonstrated that cancer cells in general oxidize a wide range of substrates in the mitochondria, even under hypoxia. Indeed, many established cancer cells have a high requirement for glutamine which also serves as the nitrogen donor in important amidotransferase reactions such as those in nucleotide [57] and UDP-GlcNAc biosynthesis, and as a source of Glu which is the most common co-substrate for aminotransferases. We have shown in SIRM studies using NMR and MS that many aspects of mitochondrial metabolism including anaplerotic pyruvate carboxylation is activated in vivo [87, 88] and ex vivo in human lung cancer tissues [82, 87]

Mitochondrial metabolism, the PPP, and glycolysis together are essential to fueling nucleotide biosynthesis, which is fundamental to cell proliferation [89]. As indicated above, the ribose subunit of the free nucleotides is directly derived from glucose (or glycogen) as shown by tracer studies with ^{13}C-glucose [85]. In contrast, the nucleobases derive from multiple sources. Uracil (and subsequently cytosine) derives primarily from aspartate, which is mainly synthesized de novo in cells and tissues as the extracellular concentration of Asp is very low and it is not efficiently transported. Asp can be synthesized via transamination of oxaloacetate with Glu in both the cytoplasm (via GOT1) and the mitochondria (via GOT2). Using [U-^{13}C,^{15}N]-Gln as the tracer, the elevated presence of the m+5 (i.e.$^{13}C_4$,$^{15}N_1$) isotopologue of Asp [25] is evidence for the activation of the aminotransferases GOT1/2 [90]. This transamination is also part of the aspartate/malate shuttle system for transferring reducing equivalents from the cytoplasm to the mitochondria for oxidation via the electron transport chain. Under respiration deficiency, pyruvate may act as an alternative electron acceptor and utilize pyruvate carboxylase to maintain Asp synthesis [91–93]. Indeed, we determined that pyruvate carboxylase is activated in vivo in human lung cancer by employing $^{13}C_6$-glucose infusion into patients coupled with SIRM analysis [87]. We also determined that Gln is a better substrate for Asp and uracil ring synthesis than glucose in cancer cells by comparing ^{13}C incorporation from $^{13}C_6$-glucose and [U-^{13}C,^{15}N]-Gln into the two products [85]. Interestingly, Asn is not a precursor of Asp in mammalian cells owing to the lack of asparaginase, but it can rescue cell growth and survival under low Gln availability [94].

Glycine-derived one-carbon metabolism plays a central role in cell cycle control during cell growth and replication [95]. Purine synthesis requires glycine as a direct donor of two carbons and one nitrogen. Glycine is also a source of the one-carbon unit in formyl tetrahydrofolate (THF) that supplies two additional purine ring carbons. Although glycine is freely available in the serum, it is also synthesized in cells from serine, which in turn is made from the glycolytic intermediate 3-phosphoglycerate. This pathway is up-regulated in some human cancers [95, 96] and as much as 8–9% of the glucose utilization may be shunted to serine synthesis [96]. In addition, purine synthesis requires Gln and we have shown that the three nitrogen atoms in the ring of purine nucleotides are derived from Gln by using [U-^{13}C, ^{15}N] Gln as a tracer. Two nitrogen atoms derive from the amidotransferase reactions and the third is from Asp, which should have received its amino nitrogen via transamination from

Gln-derived Glu [25]. Other than fueling nucleotide biosynthesis, one-carbon metabolites such as 5-Methyl THF is the source of methyl groups for replenishing S-adenosylmethionine (SAM) from S-adenosylhomocysteine (SAH). SAM is the universal methyl donor for a wide range of methyl transferases involved in epigenetic methylation of histones as well as cytosine in DNA [97].

Although many cancers are driven by alterations in oncoproteins and tumor suppressors, which often lead to reprogramming of cell metabolism, there are also cases where loss or gain of function directly in enzymes is key to carcinogenesis. For example, loss-of-function alterations in fumarate hydratase (FH) and succinate dehydrogenase (SDH) are important to the development of several aggressive familial cancers of the kidney [98–101]. Such loss of enzyme activity results in the accumulation of high levels of fumarate or succinate, respectively, which are derived predominantly from glutamine based on ^{13}C tracer studies in cell cultures [56, 102] (cf. Figure 5). Cells expressing these inactive enzymes display substantial reductive carboxylation via a reversal of the isocitrate dehydrogenase (IDH) reaction to support lipogenesis for growth. The accumulated fumarate and succinate are potent inhibitors of the α–ketoglutarate-dependent dioxygenases, including prolyl hydroxylases (e.g. those that hydroxylate hypoxia-inducible factor Hif1α for marking for proteasomal degradation) and the epigenetic modulating enzymes Ten-eleven translocation enzyme (TET) (Fig. 5) and jumonji-domain histone K/R demethylases.

In addition to loss of function enzyme variants, there are rare gain-of-function mutations in the genes encoding *IDH1* and *2*, which are NADPH-dependent cytoplasmic and mitochondrial isoforms, respectively. These mutations alter the enzyme activity from oxidative decarboxylation of isocitrate to α–ketoglutarate (αKG) to reduction of αKG to 2-hydroxyglutarate (2HG) with NADPH [103, 104]. 2HG is now known as an oncometabolite that accumulates to high levels in certain gliomas [105] and in acute myeloid leukemia (AML) [106]. This has the combined effect of depleting NADPH (which is needed both for anabolic purposes and for detoxifying excess H_2O_2 production) and αKG (which inhibits the αKG-dependent dioxygenases [107, 108]) as well as inhibiting histone demethylation to block cell differentiation [109].

NADPH is a key coenzyme and the NADPH/$NADP^+$ ratio is tightly regulated in cells to support anabolic reactions and anti-oxidative defense. The possible sources of the hydride have been elegantly determined using different deuterated substrates that result in the production of NADPD [110, 111]. When combined with genetic modification of specific enzymes, tracing deuterium (D or ^2H) can also provide information about compartmentation of different NADPH-producing activity such as PPP and reactions catalyzed by malic enzymes and IDHs [110], which prompts a need for reassessing NADPH production in cancer cells.

6. Future directions

In most cases, metabolic reprogramming in human cancer involves quantitative (i.e. altered production of existing metabolites) rather than qualitative changes (formation of new metabolites due to e.g. gain-of-function mutations such as in IDH [112]). Regardless of the

nature of the reprogrammed events, stable isotope tracing has opened many new avenues for resolving and quantifying metabolic fates in atomic detail that are crucial to deciphering reprogrammed metabolic networks required for supporting cancer cell proliferation, survival and metastasis. Tracer data are also essential for rigorous metabolic flux analysis (e.g. [113, 114]), which can inform the changes in the kinetics of individual enzymes and/or transporters in given metabolic networks in-cell, in-tissue, and in vivo, in response to disease development or therapeutic interventions. This information is valuable in its own right but is even more powerful when linked to functional genomic data for elucidating altered metabolic regulatory network [115, 116].

6.1. Cancer metabolomic studies in better preclinical models

The majority of the stable isotope tracer studies have been performed in 2D cell cultures, which cannot recapitulate proper cell-cell and cell-extracellular matrix interactions. These microenvironment factors can have a significant impact on cell behavior and functions including gene expression profiles and drug responses [117–121]. This is a key reason for the U.S. National Cancer Institute to abandon the NCI-60 cell panel for drug screening purposes. The development of 3D cell cultures including spheroids of single cell types and organoids of multiple cell types [118, 122] can circumvent these drawbacks of 2D cell cultures. Multiple 3D cell culturing techniques are now available including scaffold (e.g. Matrigel, hydrogel) and scaffold-free (hanging drop, micropatterned/U-shape cell repellent plates) methods [118, 122, 123]. They variously suffer from technical challenges such as long spheroid formation times with variable efficiency and/or difficult handling for high throughput assays [120]. For adaptation to SIRM studies, there are added challenges from matrix contamination (e.g. from Matrigel) that interferes with metabolite qunatification and/or limitations in scaling-up.

A recently developed matrix-free 3D culture method, magnetic 3D bioprinting (M3DB) can overcome these difficulties. This technique utilizes nanoparticles composed of gold, iron oxide, and poly-L-lysine to magnetize cells, followed by spheroid or organoid assembly within minutes to hours under mild magnetic forces in 6- to 384-well cell repellent plates [120].

We have employed this system in a SIRM study of a patient-derived organoid (PDO) culture subjected to [$^{13}C_6$]-Glc ± anti-cancer selenite treatments in a 96-well plate. As shown in Figure 6, [$^{13}C_6$]-Glc transformations into the metabolites of glycolysis, PPP, Krebs cycle, gluconeogenesis (GNG), and purine/pyrimidine/glutathione biosynthesis pathways were readily observed, in addition to multiple other pathways (e.g. UDPGlcNAc synthesis) of importance to cancer cell growth and development (not shown). It is also clear that this PDO was insensitive to SeO_3 treatment based on the metabolic responses, unlike A549 cells in 2D cultures with extensive growth and metabolic changes after only 24 h of 6.25 μM SeO_3 treatment [115, 124]. This study demonstrates the feasibility of performing SIRM studies in high-throughput formats on cancer cell organoids or spheroids. Future developments in this area such as SIRM coupled with 3D co-cultures of different cell types [125, 126] and microfluidics-based 3D cultures [123] will help transform our understanding on the influence of tumor microenvironment on cancer and stromal cell metabolism.

Another common preclinical model for human cancer is tumor xenografts in mice including those of established cancer cells or preferably patient-derived tumor xenografts (PDTX). Stable isotope tracer studies have been performed in vivo on mouse xenograft models via intravenous (i.v.) injection of tracers as a bolus [127] and/or continuous infusion [128]. These tracer introduction methods involve physical constraints and/or the use of anesthetics and minor surgery, which traumatize, to variable extents, the animals and thereby alter their individual baseline metabolism. In addition, the duration of the tracer infusion is typically minutes to hours, which limits the observed pathways to those with relatively rapid turnover such as glycolysis, PPP, and the Krebs cycle. Pathways that involve de novo synthesis of lipids, proteins, and nucleotides require much longer periods of tracer administration, which is problematic with the i.v. method. We have recently introduced stable isotope tracer delivery via a liquid diet, which is non-invasive with no restriction on the tracer duration [2]. Using this method, we observed de novo protein synthesis in different mouse organs from 18 h of [$^{13}C_6$]-Glc feeding (Figure 7). When applied to PDTX mouse models, we noted distinct glucose metabolism between primary tumor and its distant lymph node metastases [2]. Future applications of such tracer delivery method can greatly facilitate metabolic network analysis including flux modeling in vivo in animal models of human cancer.

6.2. High throughput multi-dimensional NMR

To greatly expand 1D NMR resolution, to gain much more structural insights, and to enhance isotopomer distribution analysis, further development in multidimensional NMR is needed, particularly in terms of the speed of data acquisition. There have been numerous developments in this area, notably non-uniform sampling [129, 130] and projection-reconstruction [131] [132] among others [133–135]. There are also hardware approaches, including the use of multiple receivers for the simultaneous acquisition of 2 or more 2D NMR data [136, 137]. The number of increments needed for multidimensional NMR can also be reduced using selective excitation methods, which could be especially useful for sugar phosphates where the spectral dispersion is low. The combination of sparse sampling in 2 dimensions [138], multiple receivers coupled with relaxation enhancement could decrease the total experimental time by more than an order of magnitude, which should enable fast-throughput high-dimensional SIRM NMR analysis in the future.

6.3. Improved quantification and isotopomer analysis by MS

Despite the very high sensitivity, mass accuracy, and resolution of UHR-FTMS, identifying and resolving different isotopologues of numerous metabolites in crude extracts remains a technical challenge. UHR-FTMS models capable of mSIRM currently range in performance from >350,000 resolution at m/z=400 (R_{400}) upwards to a few million, and systematic analysis of labeled metabolites certainly benefits from further improvement of the UHR-FTMS hardware both in terms of sensitivity and m/z resolution (e.g. R_{400} up to 10^6 for Thermo's Orbitrap Fusion™ Lumos™ and R_{400} >10^6 Bruker's SolariX XR FTMS). It is also advantageous to improve on the fragmentation methods for UHR-FTMS so that more complex, substructure-specific MS/MS data can be acquired for metabolite identification and labeled position (isotopomer) analysis. For example, the recent development in UVPD (UV photodissociation) [139] technique for UHR-FTMS enables fragmentation at fatty acyl double bonds, yielding at once double-bond positions and narrowing of stable isotope

locations. Both are traditionally tediously difficult tasks and will be a valuable addition to lipid pathway analysis in SIRM studies. Moreover, the increasing choices of stable isotope tracer standards will greatly facilitate robust MS quantification of unlabeled and labeled metabolites alike. This is synergistic with the high throughput direct-introduction UHR-FTMS methods that are undergoing both active improvements in nanoelectrospray, DESI, DART and others, and emergent techniques such laser-thermal desorption and “paperspray” among others [140]. All these (and other) developments in MS greatly extend the choice of experimental designs and tracer standards for standard addition-based quantification of labeled metabolites such as the use of deuterated amino acid standards for quantifying ^{13}C and/or ^{15}N labeled amino acids in crude extracts, anchored by UHR-FTMS [27, 29].

Ultrahigh sensitivity also means that there is an increased tendency to carry out what is really trace analysis at femtomole or lower quantities in the sample, for those low abundance but highly potent metabolites. Even moderate abundance metabolites become trace level in small samples, such as single cells [141, 142]. For example, in a cell of volume 1–2 pL, a fairly typical volume for an epithelial cancer cell, the amount of substrate present at a cellular concentration of 1 μM is 1–2 amol, which with an ionization efficiency of 1% in electrospray after chromatographic separation implies only a few thousand ions reach the detector.

6.4. Informatics needs for pathway mapping and analysis

Once the labeling patterns of metabolites are determined in tracer studies, they are valuable for rigorous reconstruction of metabolic networks, including compartmentalized events across cells and tissues [26, 55, 143, 144]. [2, 45, 66, 145–150] Although multiple databases and tools are available for pathway mapping, such as KEGG [151, 152], HMDB [153], Metacyc, Recon3D and others [154–160], automated reconstruction of atom-resolved pathways based on tracer data remains challenging [159]. There is also a general lack of delineation of pathway compartmentation, particularly in terms of tissue specificity in these databases. Databases of compartment-delineated, atom-resolved metabolic networks and tracer-friendly pathway reconstruction tools are urgently needed. Progress is being made on atom-resolved metabolic atlases and reconstructions [158, 160–163], but they are not yet ready for general use.

Accurate reconstruction of metabolic networks at the atomic level is the foundation for metabolic flux analysis, including Flux Balance Analysis (FBA) and Kinetic Modeling Analysis (KMA). The FBA approaches use reaction stoichiometries and steady state assumptions [164] such that detailed kinetic parameters of the enzymes/transporters participating in the networks are not required. The resulting flux models do not provide individual enzyme/transporter kinetics, nor can the model predict flux changes in response to perturbations of participant proteins. The KMA approaches require a full set of enzyme/transporter kinetic parameters to solve sets of differential equations either at steady state, or under non-steady state conditions [165–173]. The models established provide individual enzymes/transporter kinetics and can predict how metabolic networks respond to changes in protein components. Currently, parameterization of these sets of equations requires extensive expert input and is time-consuming. Future informatics development in assisting the

parameterization effort, e.g. narrowing the initial parameter space, is of particular importance to encourage non-expert in engaging the kinetic flux modeling approaches. The power of these approaches lies not only in quantitative understanding of reprogrammed metabolism in cancer or other human diseases but also in facilitating system level integration of reprogrammed metabolism with gene expression and proteomics changes. Such integration is fundamental to deciphering the regulatory metabolic networks and in turn gaining mechanistic insights into disease progression and therapeutic efficacy.

By overcoming the multitude of educational and technical challenges that currently limit the experimental, analytical, and informatics tools already in place and under development, we fully expect that the application of stable isotope tracers to metabolic research in cancers or other diseases will greatly expand and flourish in the future.

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Abbreviations:

1D, 2D, 3D	one-, two-, three-dimensional
ECF	ethyl chloroformate
HSQC	heteronuclear single quantum coherence
mamu	milli atomic mass units
PPP	Pentose Phosphate Pathways
QDA	*N*-(2-^{15}*N*-aminooxyethyl)-*N*,*N*-dimethyl-1-dodecylammonium
QDE	*N*-(2-^{15}*N*-iodoacetamido)-*N*,*N*-dimethyl-1-dodecylammonium
TOCSY	Total Correlation Spectroscopy
SIRM	Stable Isotope Resolved Metabolomics
UHR-FT-MS	ultrahigh resolution Fourier transform mass spectrometry

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Highlights

- Stable isotope resolved metabolomics maps and quantifies metabolic networks
- Stable isotope tracing tracks known and novel reprogrammed metabolism
- NMR and mass spectrometry complement, cross-validate, and maximize isotope distribution analysis in metabolites

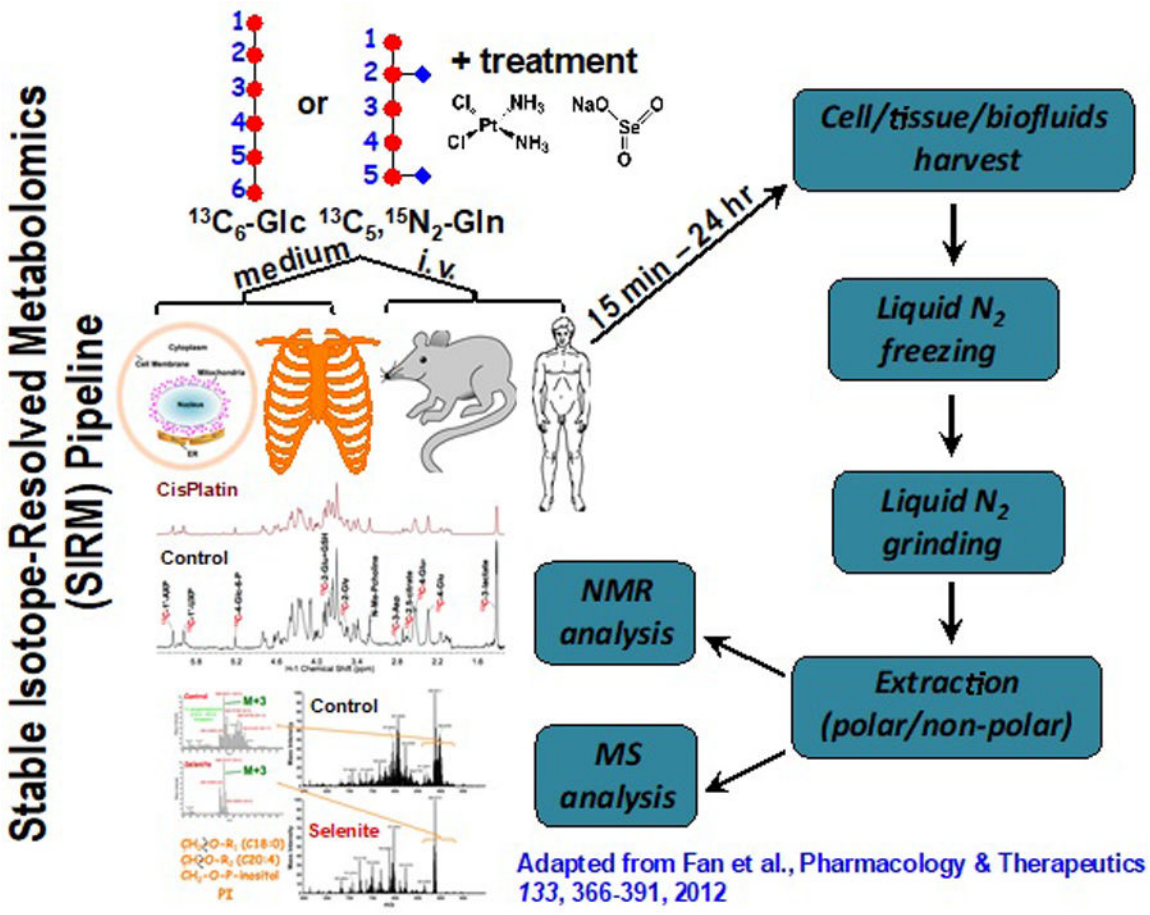

Figure 1. SIRM Pipeline.

Stable isotope-enriched precursors are introduced to the biological system for a defined period, followed by harvesting the tissue, biofluid or medium, metabolite extraction and analysis by MS and NMR. The isotopomers and isotopologue distributions are then incorporated into metabolic network maps and flux models. Such results may also be combined with genomics, proteomics and in vivo NMR information acquired on the same samples.

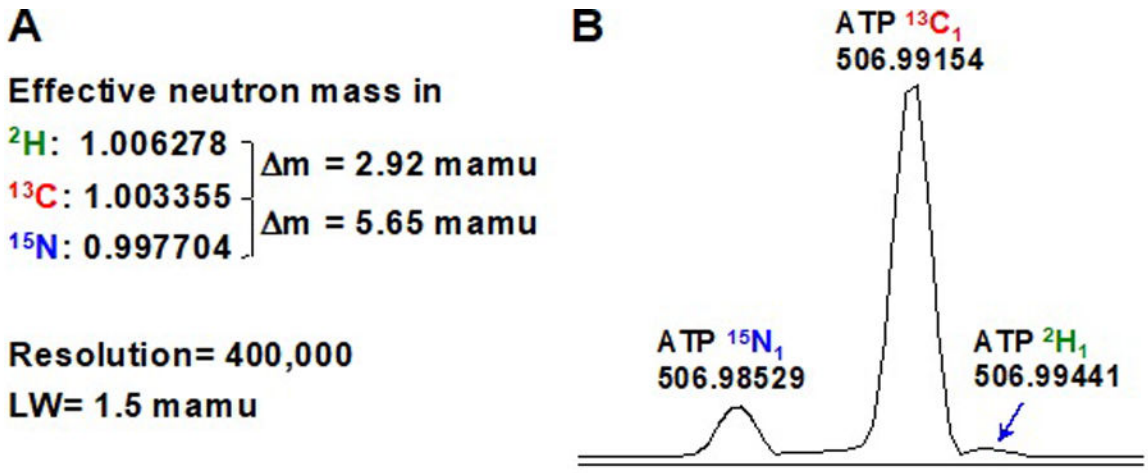

Figure 2. UHR-FTMS distinguishes isotopes of identical nominal mass.

Panel **A** indicates the m/z difference between ^{2}H and ^{13}C or ^{13}C and ^{15}N in the mamu range as well as the expected resolving power of a commercial FTMS instrument. Panel **B** shows the resolution of ^{2}H, ^{13}C, and ^{15}N in ATP in a crude cancer cell extract (adapted from [3]). LW is the width at half height in milli atomic mass units (mamu).

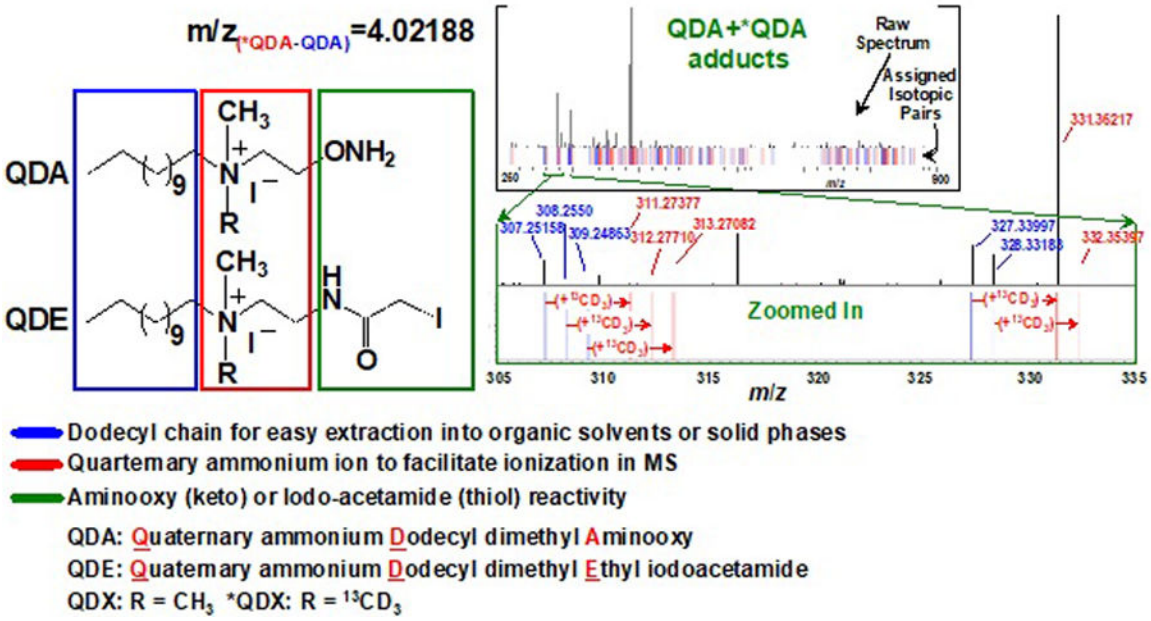

Figure 3. Chemoselective probe design for facilitating MS-based structural analysis and quantification.

The probe design includes the carbonyl-selective aminooxy for QDA [1] or thiol-selective iodoacetamide groups for QDE [4], plus two common structural motifs of a dodecyl chain for extractability into organic phases and a quaternary ion for ready ionization in MS. Each probe can also be prepared as a pair with R equal to CH_3 and $^{13}CD_3$ (*QDA or *QDE). The resulting adducts of metabolites with a 1:1 mixture of QDA and *QDA can be readily discerned by an *m/z* difference of 4.02188 by UHR-FTMS, as illustrated by a “bar-code”-like profile of the adduct pairs in the spectrum on the top right (adapted from [1]).

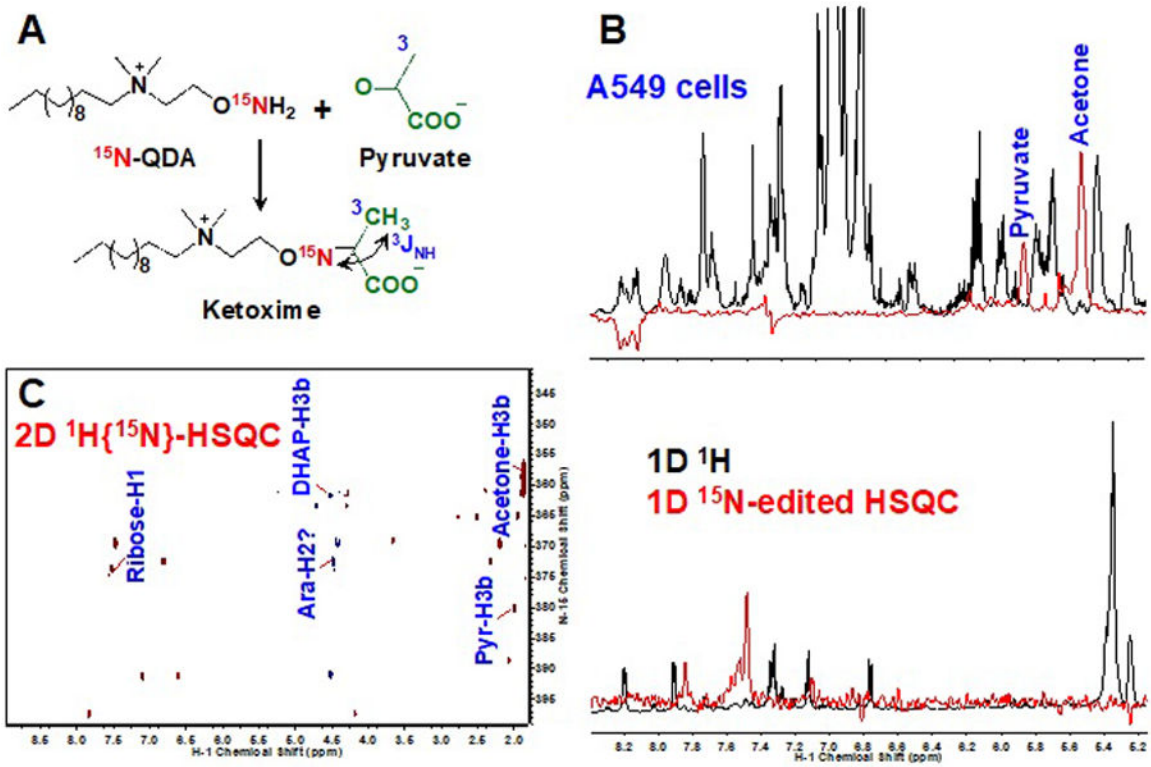

Figure 4. Chemoselective derivatization enables NMR detection of carbonyl metabolites.

The QDA probe from Figure 3 was modified to contain a ^{15}N aminooxy group, which forms a ketoxime adduct upon reaction with carbonyl metabolites such as pyruvate (**A**). The resulting three-bond coupling between ^{15}N and neighboring protons of carbonyl metabolites enables their detection in crude A549 cell extracts by 1D (**B**) and 2D (**C**) ^{15}N-edited HSQC experiments. Also shown in **B** is the comparison of unedited ^{1}H and ^{15}N-edited HSQC spectra with clear detection of carbonyl metabolites in the latter but not in the former

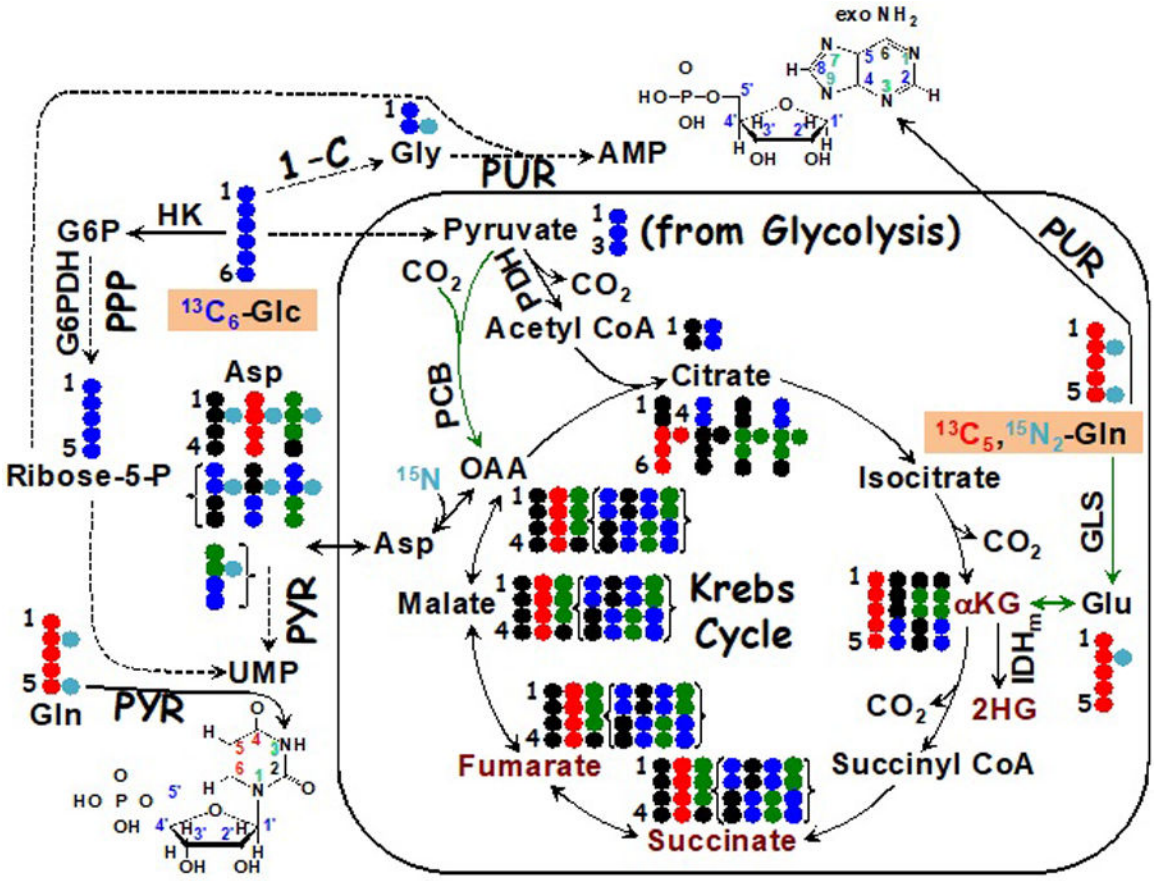

Figure 5. Metabolism and epigenetic regulation.

The diagram depicts ^{13}C tracing from $^{13}C_6$-glucose (●) or $^{13}C/^{15}N$ tracing from $^{13}C_5$,$^{15}N_2$ glutamine (●/●) to the synthesis of nucleotides and effectors (dark red text) involved in epigenetic modifications, including αketoglutarate (αKG), 2-hydroxyglutarate (2HG), succinate, and fumarate. *IDH1/2* mutants produce the histone demethylation inhibitor 2HG from αKG while inactivating *SDH* and *FH* mutations generate high levels of succinate and fumarate, which are inhibitors of αKG-dependent dioxygenases including TET involved in demethylation of methylcytosine in DNA. Green arrows denote anaplerotic inputs into the Krebs cycle mediated by pyruvate carboxylase (PCB) (●) and glutaminase (GLS) reactions. { } encloses scrambled ^{13}C labeling patterns of succinate, fumarate, malate, and Asp after one turn of the Krebs cycle. ●: ^{12}C; OAA: oxaloacetate; HK: hexokinase; G6PDH: glucose-6-phosphate dehydrogenase; PDH: pyruvate dehydrogenase; IDH_m: isocitrate dehydrogenase mutant; PUR: purine synthesis; PYR: pyrimidine synthesis; PPP: pentose phosphate pathway; 1-C: one-carbon pathway; exo: exocyclic.

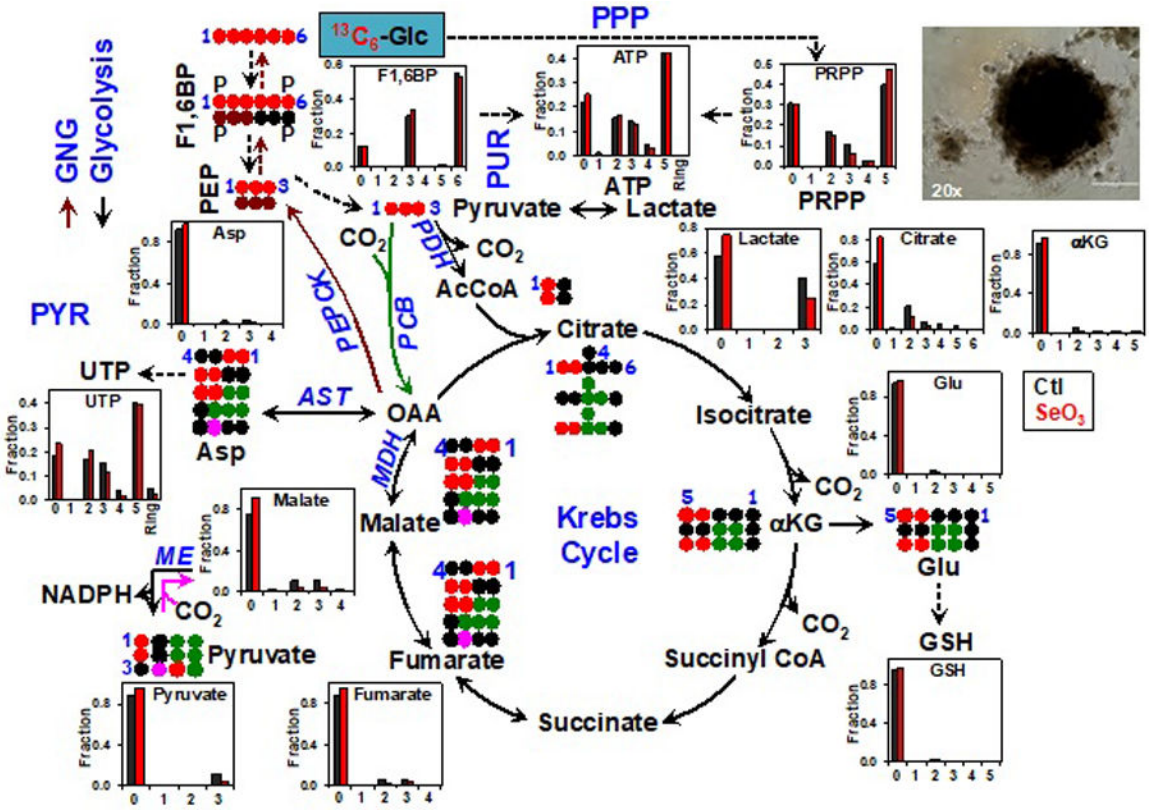

Figure 6. SIRM study on patient-derived organoids (PDO) in response to anti-cancer selenite.

PDO was isolated from a non-small cell lung cancer patient (UK022) tumor xenograft in Matrigel and subsequently grown as M3DB cultures (example 20x image shown) in [$^{13}C_6$]-Glc+DMEM medium ± 10 μM selenite (SeO_3) for 48 hr. Atom-resolved tracing from [$^{13}C_6$]-Glc to glycolytic, Krebs cycle, and gluconeogenic (GNG) metabolites is shown along with the ^{13}C labeling patterns of these metabolites in response to SeO_3. Also shown are the SeO_3-induced changes in ^{13}C labeling patterns of the products of PPP, purine (PUR)/ pyrimidine (PYR) synthesis, and glutathione (GSH) synthesis. The numbers in X-axis represent the number of ^{13}C atoms (isotopologues) in each metabolite while “Ring” in ATP and UTP indicates the sum of all ^{13}C-nucleobase isotopologues. ➔ ➔ denote GNG and anaplerotic inputs into the Krebs cycle mediated by PCB, respectively. ●: ^{12}C; ●,●,●: PDH-, PCB-, or malic enzyme (ME) initiated Krebs cycle reactions, respectively; ●: GNG; F1,6BP: fructose-1,6-bisphosphate; PEP: phosphoenolpyruvate; PRPP: phosphoribosylpyrophosphate; PEPCK: PEP carboxykinase; MDH: malate dehydrogenase; AST: aspartate amino transferase. All other symbols and abbreviations are as in Fig. 5.

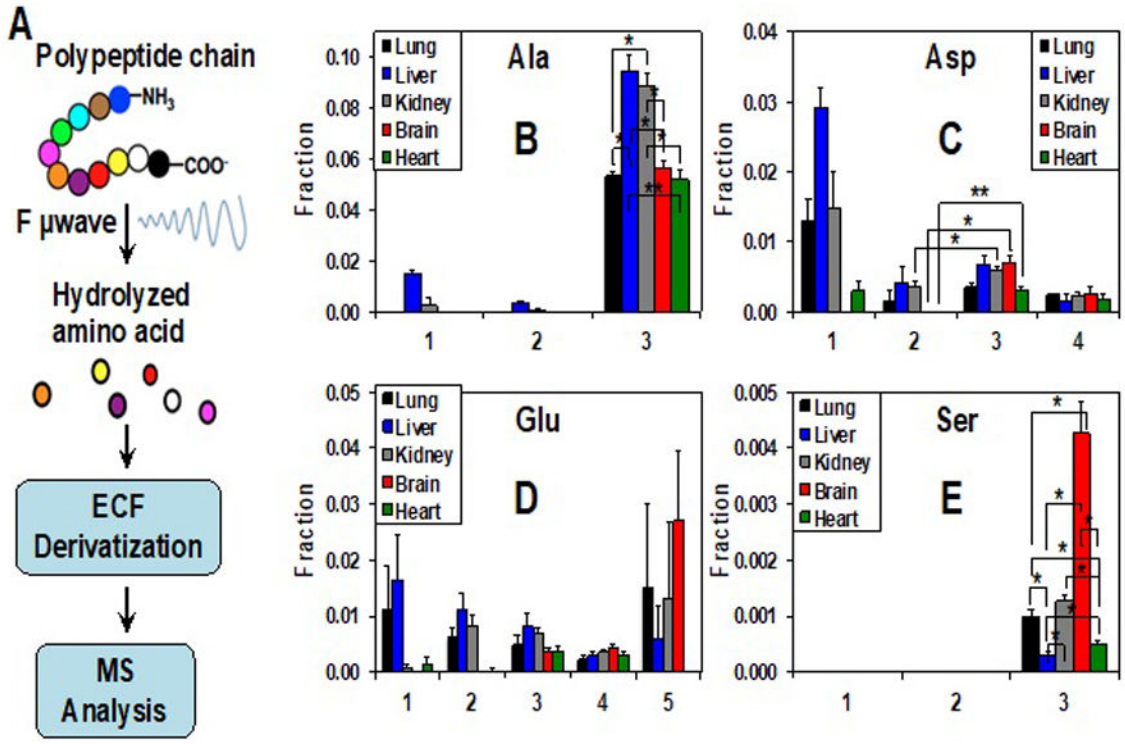

Figure 7. ECF-UHR-FTMS analysis indicates differential [$^{13}C_6$]-glucose incorporation into proteins in 5 mouse organs.

Extracted tissue proteins were hydrolyzed in 6N HCl by focused-beam microwave (**F μwave**) digestion. The free amino acids liberated were derivatized with ethyl chloroformate (ECF) before analysis by UHR-FTMS (**A**). Fractional enrichment distribution of ^{13}C isotopologues of the proteinaceous amino acids Ala, Asp, Glu, and Ser is shown in **B** to **E**, respectively. The x-axis represents the number of ^{13}C atoms present in each isotopologue. Values shown were mean ± SEM (n=3). * and ** in **B**, **E** denote false discovery q values for liver or kidney versus lung, brain, and heart; 0.01<q<0.05 and 0.001<q<<0.01, respectively. * in **C** denotes p values (<0.05–0.01) for $^{13}C_3$- (**3**) versus $^{13}C_2$-Asp (**2**) for kidney, brain, and heart (reprinted from Fig. S6, ref [2]. under a Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/).

Table 1.

Common stable-isotope enriched compounds and their uses

Tracer	Pathways sampled	references
[U-^{13}C]-glucose	glycolysis, Krebs cycle, PPP, glycogen synthesis, hexosamine biosynthesis pathway, serine-glycine-one carbon metabolism, nucleotide and lipid synthesis	[10, 45]
[^{13}C-1,2]-glucose	Non-oxidative versus oxidative branches of the PPP	[46, 47]
[^{13}C-3,4]-glucose	anaplerosis via pyruvate carboxylation	[48, 49]
[^{13}C-1]-pyruvate	Lactic fermentation	[50]
[U-^{13}C]-lactate	Krebs cycle, gluconeogenesis	[51–54]
[U-^{13}C,^{15}N]-glutamine	glutaminolysis, Krebs cycle, gluconeogenesis, transamination, nucleotide and lipid synthesis	[55–59]
[U-^{13}C]-palmitate [U-^{13}C]-octanoate [U-^{13}C]-oleate	beta-oxidation, fatty acid synthesis, fatty acid uptake	[41, 60–62] [63]
[U-^{13}C]-serine	Serine and one-carbon metabolism; purine biosynthesis; lipid synthesis (PS, sphingolipids)	[64] [65]
$^{2}H_3$-serine	Serine and one-carbon metabolism; purine biosynthesis; lipid synthesis (PS, sphingolipids)	[29]
[U-^{13}C]-glycine	Purine biosynthesis; glutathione biosynthesis	[65, 66]
^{13}C branched chain amino acids	Amino acid metabolism	[67–69]
^{15}N arginine, citrulline	Arginine/NO metabolism	[70]
[U-^{13}C]-glycerol	Lipid synthesis, gluconeogenesis-PPP interactions	[41, 71, 72]
$^{2}H_2O$	Lipid synthesis in vivo	[73, 74]

See also Cambridge Isotope Laboratories (CIL) note: MET_RSCH_CANCER (2/13/18)