Metabolic Biomarker and Kinase Drug Target Discovery in Cancer Using Stable Isotope-Based Dynamic Metabolic Profiling (SIDMAP)

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Abstract: Tumor cells respond to growth signals by the activation of protein kinases, altered gene expression and significant modifications in substrate flow and re-distribution among biosynthetic pathways. This results in a proliferating phenotype with altered cellular function. These 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. Novel drugs effective in particular cancers exert their anti-proliferative effects by inducing significant reversions of a few specific non-oxidative anabolic pathways. Here we present evidence that cell transformation of various mechanisms is sustained by a unique disproportional substrate distribution between the two branches of the pentose cycle for nucleic acid synthesis, glycolysis and the TCA cycle for fatty acid synthesis and glucose oxidation. This can be demonstrated by the broad labeling and unique specificity of [1,2-$^{13}C_2$]glucose to trace a large number of metabolites in the metabolome. Stable isotope-based dynamic metabolic profiles (SIDMAP) serve the drug discovery process by providing a powerful new tool that integrates the metabolome into a functional genomics approach to developing new drugs. It can be used in screening kinases and their metabolic targets, which can therefore be more efficiently characterized, speeding up and improving drug testing, approval and labeling processes by saving trial and error type study costs in drug testing.

INTRODUCTION

Although the gene modulating effects of a great number of signaling mechanisms and protein kinase activities have been described, too many details of how they exert their controls on cell metabolism and their influence on cell phenotype are still obscure [1, 2]. In order to understand gene function and the role of kinase-based signaling cascades, research efforts are increasingly exploiting metabolomics. This discipline analyzes changes in the levels of substrates, intermediary metabolites and products in order to reveal metabolic adaptive changes that are the consequences of function modulating events in cells or organisms. In general, variations and changes in components of the metabolome closely reflect adaptation of an organism to its microenvironment as defined by substrate availability, intermediate synthesis and macromolecule product formation [3]. The major regulatory components of cell function, namely the genome, transcriptome and proteome, ultimately act on the metabolome by the altered expression of substrate transporter proteins and metabolic enzymes, which strongly influence pathways of biosynthetic processes as well as energy production. The close interactions among these components establish a rationale for integrating functional genomics, proteomics and metabolomics, as a means to study complete intracellular signaling processes that regulate phenotype and cellular function [4]. Due to the well known altered metabolic characteristics of tumor cells that include the activation of non-oxidative anabolic processes, studying clonal tumor cell metabolomes can reveal novel metabolic biomarkers of cell transformation, specifically characterize kinases and elucidate drug targets with metabolic actions beyond the influence of genes and protein targets.

A significant limitation in characterizing kinase actions and understanding their biological function is the lack of assays evaluating signal specific cellular metabolic events downstream of the anticipated changes in gene expression and protein phosphorylation. Although the levels of protein phosphorylation triggered by kinases can readily be measured by changes in intracellular ATP and ion concentrations in high throughput screening assays, the information obtained does not reveal specific cellular metabolic events as a result of multiple enzyme protein phosphorylations by the kinase being screened. The rates of cell proliferation, hormone secretion or apoptosis formation need additional tests in cell based systems, where the cell itself remains a “black box” in terms of a mechanistic understanding of kinase action through metabolic adaptive changes to new phenotypes including necrosis or apoptosis formation. For example, the kinase inhibitory agents KT 5720, rottlerin and quercetin were found to inhibit many protein kinases, sometimes much more potently than their

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presumed targets, and therefore conclusions drawn from their use in cell-based experiments are likely to be erroneous [5]. These results demonstrate that simply studying their effect on kinases that are closely related in primary structure and function cannot assess the specificities of protein kinase inhibitors.

Kinases impact least four levels of regulation that modify cell function, phenotype and drug response. Together they provide a better understanding of an organism from a functional genomics perspective. These steps include:

1) Signal initiated, receptor-dependent kinase based cascades and transcription factors which influence,

2) the expression of genes and the transcription of specific *m*RNAs;

3) biosynthesis of proteins and their kinase based modifications and

4) changes in the levels, synthesis rates, exchange and utilization of low and high molecular weight intermediates and product bio-molecules comprising the metabolome.

The last step of an organism's adaptive response to growth modifying signaling events always involves modifications in macromolecule synthesis rates, especially that of RNA, DNA fatty acids and proteins. This is ultimately reflected in the tumor cell by an increase in metabolite flow through individual branches of the pentose cycle, primarily that of the non-oxidative branch, but also decreased substrate utilization for energy production and fatty acid synthesis. Functional genomics studies, until recently, have mainly used gene expression arrays and proteomics studies, most commonly in primitive organisms [6]. These approaches do not necessarily elucidate the downstream effects of gene expression involving the metabolome well enough to establish novel drug targets or biomarkers for anti-kinase drug efficacy testing in more complicated cancer cells. Such cells exhibit extreme genetic instability and thus complex variations in their genetic profiles, especially in undifferentiated advanced progenies. Because metabolic profiling provides otherwise unobtainable information about post-genomic events involving the metabolome, it is an irreplaceable tool in a comprehensive multidisciplinary approach to functional genomics.

There are two basic approaches in the literature to metabolome research focused on response to growth altering signaling events. Most studies measure the accumulation or clearance of narrowly defined metabolites from particular pathways in primitive organisms, mainly plants, yeast and bacteria [7-12]. Other investigations have centered on diet-dependent changes in mammalian systems involving the serum metabolome (metabolic serotype) [13-15]. These approaches provide useful information, but leave unmet several important challenges that must be overcome to provide a complete characterization of the metabolome. First, although metabolite levels can easily and accurately be measured, their precursors and synthesis pathways remain elusive and in many instances there exist alternative synthesis pathways for the production of the same metabolite within the metabolome. Therefore gene mutations and kinase based modification of gene expression, enzyme protein synthesis and protein modifications often yield "silent" phenotypes and diseases [16]. Without metabolic profiling, signal and kinase-initiated adaptive responses, their molecular targets and the mechanisms of drug action often remain poorly understood and confusing from the functional genomics point of view [17].

In the past two decades genetic and proteomics studies have utilized and advanced highly specific labeling technologies (PCR, ^{32}P, precipitating antibodies, immunohistochemistry, etc.). An analogous approach will benefit metabolome research, new drug discovery and the testing of kinases and their inhibitors for metabolic consequences of their upstream actions. This review evaluates tracer methods and applications of a stable isotope-based dynamic metabolic profiling approach, broadly applicable for metabolic profiling of various organisms, kinase-based metabolic changes and anti-kinase drug actions in cancer. The technique utilizes the principles of accumulation, exchange, dilution and rearrangements of specific stable isotope labeled atoms in bio-molecules during synthesis and enzymatic modifications. It is particularly suited for studying healthy and diseased conditions as well as protein kinase-induced signal dependent overall metabolic effects in mammalian systems for drug development [18]. The potential applications of specifically labeled stable isotope precursors are immense and include practically all areas of biomedical research and diagnostics, especially that of cancer, where constitutively active kinase cascades play a pivotal role in tumor formation, disease progression and response to therapy. Yet, the most promising utility of the stable isotope-based dynamic metabolic profiling (SIDMAP) technology is its ability to integrate the crucial information contained within the metabolome into the functional genomics concept of bio-medical research. That integration allows researchers to add to genomics' and proteomics' descriptions of what cells *can* and *may* do, the understanding provided by metabolic profiling of what cells *actually* do under specific and fluctuating macro- and microenvironmental conditions as determined by growth signals and their interruptions by kinase targeting drugs.

STABLE ISOTOPE LABELED SUBSTRATES AS TRACERS IN METABOLIC STUDIES

Early studies that utilized stable isotope labeled precursors, such as deuterated water or ^{13}C labeled substrates, and their incorporation into various intermediates were performed to study catabolic reactions in various organisms [19, 20]. These studies clearly demonstrated that isotope labeled precursors are biologically active in bacteria [21], plants [22] and mammalian cells [23], and that they possess great advantages in studying complex biochemical networks. Several isotope designs and substrates have been developed for studies of biological processes, which often are affected by signal-induced kinase-based cellular cascades. Gas chromatography/mass spectrometry (GC/MS) has mainly provided the instrument basis for stable isotope based metabolic studies of plasma amino acid synthesis and turnover rates in order to determine ^{15}N enrichments of glycine and alanine in animals in different metabolic states. This technology elegantly demonstrated that isotope-

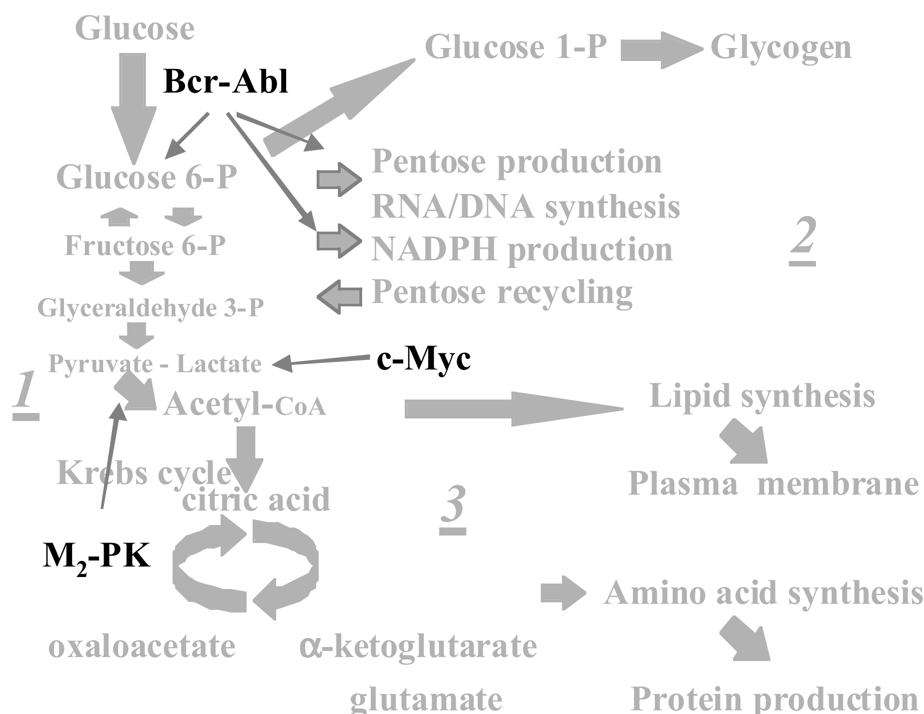

Fig. (1). Interconnected metabolic pathways and known targets of growth signaling cascade kinases. Glucose broadly utilized in tumor cells readily labels major metabolite pools (*1* glycolysis; *2* pentose cycle; *3* TCA cycle) either through as a direct substrate or through carbon exchange reactions. The specificity for metabolic pathway substrate flow measurement is provided by the loss, dilution and rearrangements of the label from $[1,2\text{-}^{13}C_2]$glucose via various reactions that yield intermediates and products with different label patterns as described in reference [18, 37]. Signal dependent or constitutively active kinases influence metabolism by activating enzymes of the glucose phosphorylation and oxidation processes in the pentose cycle (BCR/ABL) or inhibiting triose oxidizing enzymes (M_2-PK) [37] or glycolysis (c-Myc) [38] ("BCR" Breakpoint Cluster Region, "ABL" Ableson leukemia virus, "M_2-PK" M_2 iso-type Pyruvate Kinase).

enrichment time-decay curves of plasma amino acids are linear over the course of the measurements after intravenous administration of a single dose of ^{15}N-amino acid [24]. It has also been demonstrated that oncogenic signals induce well defined changes at specific metabolic sites that can be studied by various label designs targeting nucleic acid precursor synthesis [25], glycogen production [26, 27], gluconeogenesis [27-29], glycolysis and pentose cycle substrate flow [30, 31], the TCA cycle [32, 33], amino acid/protein synthesis [34] and fatty acid metabolism [35, 36] as shown in Fig. (**1**). Although metabolic processes are inherently complex, the overall effects of signaling cascades and kinase activity altering drugs induce specific changes in the metabolome. Such changes are very substantially involved in the cellular adaptation process to growth altering signals, cell cycle arrest or the induction of apoptosis.

Dynamic metabolic profiling determines specifically labeled glucose flow towards *lactate*, *glycogen*, *glutamate*, nucleic acid *ribose/deoxyribose, palmitate*, *stearate* and *oleate* syntheses, as well as the release of CO_2 in cell cultures, animals or humans. Thus, it indicates specific changes in glucose utilization for biomolecule synthesis in glycolysis, the pentose cycle, TCA cycle and fatty acid synthesis pathways. The basis of stable isotope based metabolic profiling is that $[1,2\text{-}^{13}C_2]$glucose metabolism produces reaction-specific isotope-labeled intermediary metabolite species, also called mass isotopomers through label incorporation, carbon exchange, isotope dilution or isotope loss. All of these isotopomers are distinctively formed by specific enzymatic reactions, making metabolic profiling an excellent tool to track detailed metabolic substrate flow changes that reflect kinase effects and responses to drugs. For detailed descriptions of the method, tracer and instrumentation we direct attention to references [18] and [37].

The scheme of a cell culture experiment for a metabolic profiling study of testing kinase effects is given in Fig. (**2**). Control (untreated) cells are incubated in the presence of the isotope labeled glucose substrate in order to generate a reference metabolic profile, to which treated test cultures are compared using stable isotope distribution patterns. One metabolic profiling experiment can reveal a comprehensive metabolic response of adaptive changes to increasing doses of a kinase inhibitory drug or gene modification, which are then compared to the reference kinase negative control cultures by collecting the following information:

1. Glucose uptake and lactate release (chemistry analysis of the culture media)
2. Direct glucose oxidation relative to glycolysis (lactate m1/m2 ^{13}C ratios).

3. Direct glucose oxidation/non-oxidative ribose synthesis for RNA/DNA nucleotide syntheses (m1/Σm versus m2/Σm, m3/Σm and m4/Σm).
4. Glucose oxidation in the pentose and TCA cycles ($^{13}CO_2/^{12}CO_2$ ratios).
5. TCA cycle anaplerotic flux (glutamate m2/m1 ratios).
6. De novo fatty acid palmitate synthesis (^{13}C enrichment in palmitate).
7. Chain elongation into stearate (^{13}C enrichment in stearate and the isotope distribution ratios in palmitate and stearate).
8. Acetyl-CoA glucose carbon enrichment (palmitate m4/m2 ^{13}C ratios).
9. Direct and indirect glycogen synthesis (glycogen m1/m2 ratios).
10. Gluconeogenesis (ratios of [1,2-$^{13}C_2$]glucose and other recombined isotope labeled glucose species in the media).

Using stable isotopes to track and elucidate changes in tumor cell cultures in response to cell transforming kinases and in response to cancer growth-controlling compounds are two examples of how metabolic profiling can benefit drug discovery. The utility of the stable isotope methodology of studying tumor cell physiology in the context of signaling events and the function of genetic background is one of the new advances in metabolome research with wide applicability. Many effective anti-cancer therapeutics limit carbon flow toward nucleic acid synthesis and shift glucose toward oxidation or fatty acid synthesis through specific metabolic reactions. Those changes make the reactions that cause them and the enzymes involved in them very suitable as new biomarkers for screening potential drugs to treat cancer or to determine if cell transformation takes place in response to a kinase-activating signal. One can view entire new dimensions in biomarker discovery and gene function analysis in cancer by looking through the lens of the stable isotope tracer labeled metabolome or SIDMAP to learn exactly how the proliferative undifferentiated tumor cell phenotype correlates with corresponding and tumor specific anabolic macromolecule synthesizing cellular metabolic events. Such knowledge can point out reliable enzymatic target sites to reverse the metabolic profiles of malignant undifferentiated cells to that of normal ones.

The question remains as to whether there is any advantage of using SIDMAP over other cell proliferation/ differentiation assays via genetic or protein markers? The answer relies on the specificity of SIDMAP capable of differentiating among interconnected metabolic pathways and their branches for macromolecule synthesis. Unlike classical

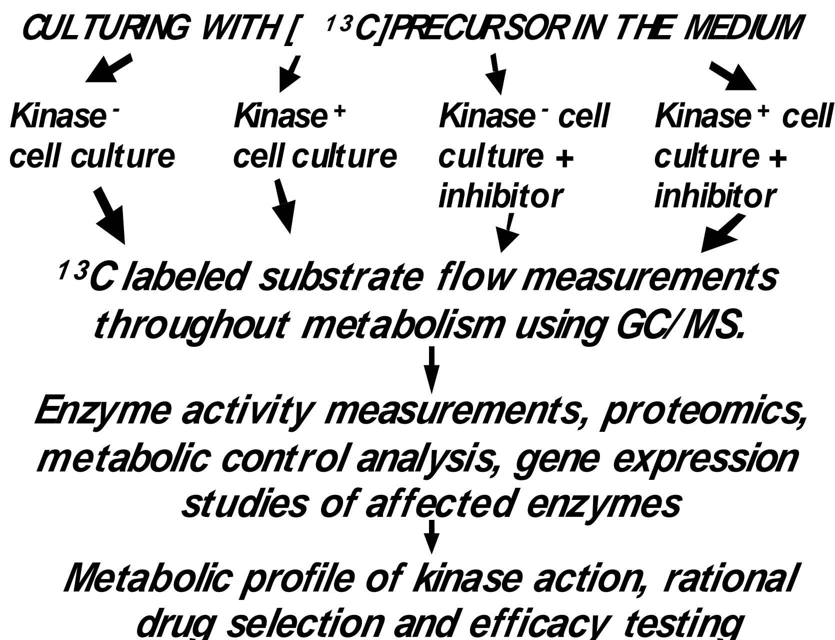

Fig. (2). Cell culture study design for stable isotope-based metabolic profiling in the presence of a kinase and a test inhibitory drug as well as the tracer. Usually three increasing doses of the test drug in 10-fold increments are applied in target kinase positive ($^+$) and control kinase negative ($^-$) cell cultures for a desired period of time (usually 48 or 72 hours), upon which metabolic profiles are compared with untreated control cultures. Control cultures contain the untreated kinase negative and kinase positive cells and the highest concentration of the vehicle for the drug. Cell pellets and the culture media are collected and ^{13}C distribution, loss, dilution and rearrangements are determined using liquid or gas chromatography/mass spectrometry (LC/MS, IRMS, NMR or GC/MS). If necessary, protein analyses, metabolic control analysis or the expression of metabolic enzymes and determination of their activities will follow for pathways where significant substrate flux changes were observed after metabolic profiling. The pharmaceutical industry and academia both benefit from characterizing kinases more efficiently, as this leads to better drug target selection, accelerated approval and improved drug labeling.

proliferation assays using various DNA labeling and cell counting techniques, SIDMAP provides not only information with regard to *de novo* macromolecule synthesis rates but also provides substantially more data through the identification of enzymatic reactions that participate in phenotype specific metabolic processes. There are numerous differences in metabolic pathway activities between tumor cells and their rapidly dividing normal counterparts such as bone marrow cells or the epithelia. For example, SIDMAPs of transformed cells in comparison with normal rapidly dividing cells reveal significant differences in glucose uptake and the utilization of oxidative versus non-oxidative pentose cycle reactions. Transformed cells heavily depend on non-oxidative ribose synthesis, TCA cycle anaplerosis, intense lactate and *de novo* fatty acid synthesis but produce very little glycogen, partially known as the Warburg effect [38]. On the contrary, rapidly proliferating normal cells rather oxidize glucose directly in the pentose and TCA cycles, utilize plasma fatty acids for chain elongation/desaturation and readily build glycogen. Accordingly, cultured normal cells subjected to SIDMAP often show no label accumulation in fatty acids and very different ^{13}C mass isotopomer distribution in RNA and DNA compared to that of transformed cells. The utility of SIDMAP in *in vivo* animal studies using the [1,2-$^{13}C_2$]glucose tracer has recently been demonstrated with great specificity and applicability to characterize complex intermediary metabolic networks in experimental animals [39]. SIDMAP also reveals drug specific metabolic effects and detailed mechanisms of anti-proliferative action in the metabolome, which are given in details below.

TRANSFORMED UNDIFFERENTIATED CELLS CAN BE IDENTIFIED BY A METABOLIC PROFILE OF INCREASED NON-OXIDATIVE PENTOSE CYCLE CARBON FLOW

Progression of certain cancers is enhanced by the autonomous growth promoting tyrosine kinase signaling ligand transforming growth factor-β (TGF-β_2) [40]. This process primarily depends on non-oxidative glucose conversion into ribose as the end-result of this signaling cascade [41]. Similarly, the acetyl-cholinesterase inhibitor and carcinogen pesticide, isofenphos, directs glucose carbon flow toward nucleic acid ribose synthesis in myeloid cells [42], resulting in a proliferative undifferentiated aneuploid cell phenotype with rapid progression into blast phase terminal leukemia [43]. The SIDMAP of these two cancer-promoting substances reveals different signaling and kinase cascades, yet the effect of these agents on the metabolome is indistinguishable, indicating a primacy of carbon distribution and non-oxidative ribose/deoxyribose synthesis in the cell transformation process (Fig. **3**).

There are similar important metabolic profile changes in the BCR-ABL kinase bearing transformed cells, consistent

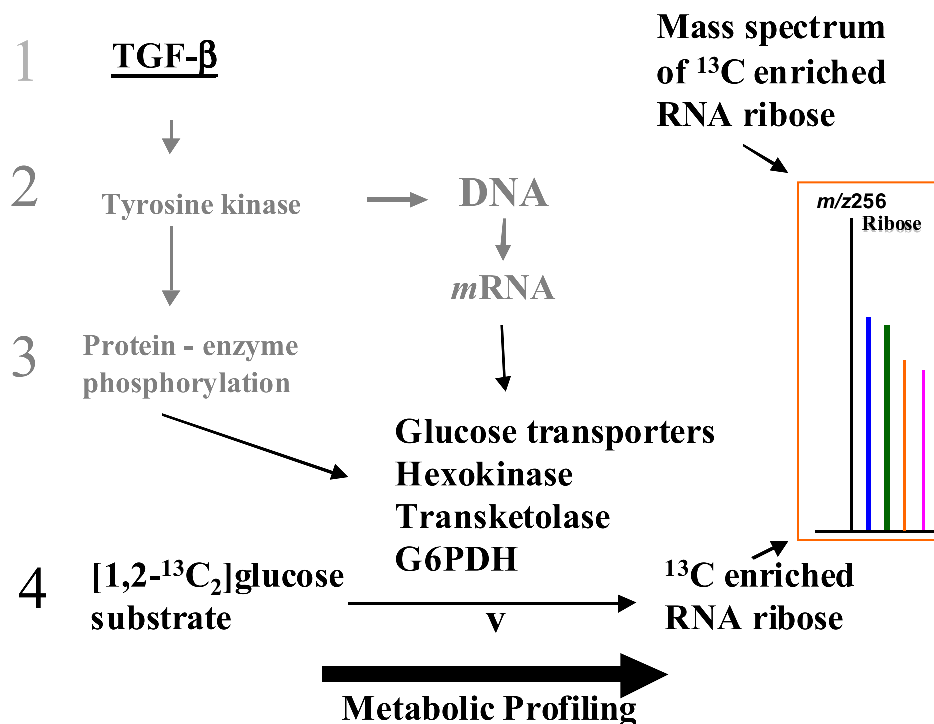

Fig. (3). Mass spectrum of ribose isolated from kinase activator (TGF-β) treated tumor cells after metabolic profiling. Biomolecules of the mammalian cell metabolome are readily labeled by [1,2-$^{13}C_2$]glucose and all intermediary metabolites of glucose, including nucleic acid ribose, become labeled by ^{13}C on various positions as described elsewhere [18, 37]. the majority of ^{13}C label from [1, 2-$^{13}C_2$]glucose accumulates in the RNA and DNA of transformed cells indicating a high rate of *de novo* nucleotide synthesis. The mass spectrum also reveals high rate of non-oxidative ribose synthesis due to the intense appearance of m2, m3 and m4 peaks, represented in this order by bars, on the mass spectrum (original data is reported in reference [41] and [42]).

with but beyond those suggested by the more limited information revealed in gene expression and enzyme protein synthesis patterns. Haemopoietic cells transfected with BCR-ABL express GLUT1, a high affinity glucose transporter. Increased glucose uptake is the metabolic hallmark of the transformation of such cells [44]. This transformation also involves the activation of subsequent glucose metabolizing enzymes, hexokinase-II and glucose-6-phosphate dehydrogenase (G6PDH) [45]. Cell-transforming agents uniformly induce carbon flow changes consistent with increased pentose cycle metabolism and the diversion of glucose carbons towards nucleic acid precursor synthesis. Therefore, the primary direction of glucose carbon flow in transformed human cells is toward RNA and DNA via the nonoxidative steps of the pentose cycle as the underlying metabolic defect in tumors cells, which thereby maintain high rates of proliferation in an unlimited fashion. The phenotypic consequences include rapidly proliferating, poorly differentiated cells with aneuploidy.

This phenotype is also supported by the oncogenic expression of the dimer M_2 isoform of pyruvate kinase (M_2PK, Fig. **2**) [46]. The oncogenic transition of metabolic gene expression lowers the affinity of substrate phosphoenol-pyruvate for pyruvate kinase decreasing glycolytic flux but expanding all glucose phosphate intermediary metabolites that promote nucleotide synthesis in the non-oxidative branch of the pentose cycle. Because it can track the changes described above, the SIDMAP of a tumor cell reveal specific pathway activities and points out metabolic enzymes as proteomics biomarkers of malignant cell transformation and growth which can be utilized in the early cancer diagnosis and drug target discovery processes. The experienced mass spectrometrist distinguishes metabolic phenotypes by the appearance of spectral changes of critical macromolecules, such as nucleic acid ribose isotopomers in the serum metabolome, produced by transformed cells and determines drug efficacy and tumor burden. These tasks as well as early cancer diagnosis can be performed based on specific enzyme reactions that indicate unusual, tumor specific mass isotopomer ratios of intermediary metabolites in blood plasma or serum.

DECREASED PENTOSE CYCLE CARBON FLOW AS A CELL DIFFERENTIATING AND GROWTH CONTROLLING MECHANISM

The expression of a constitutively active tyrosine kinase signaling protein as a result of the re-alignment of the breakpoint cluster region and Ableson leukemia virus proto-oncogene sequences (Bcr/Abl) is the basis of oncogenic

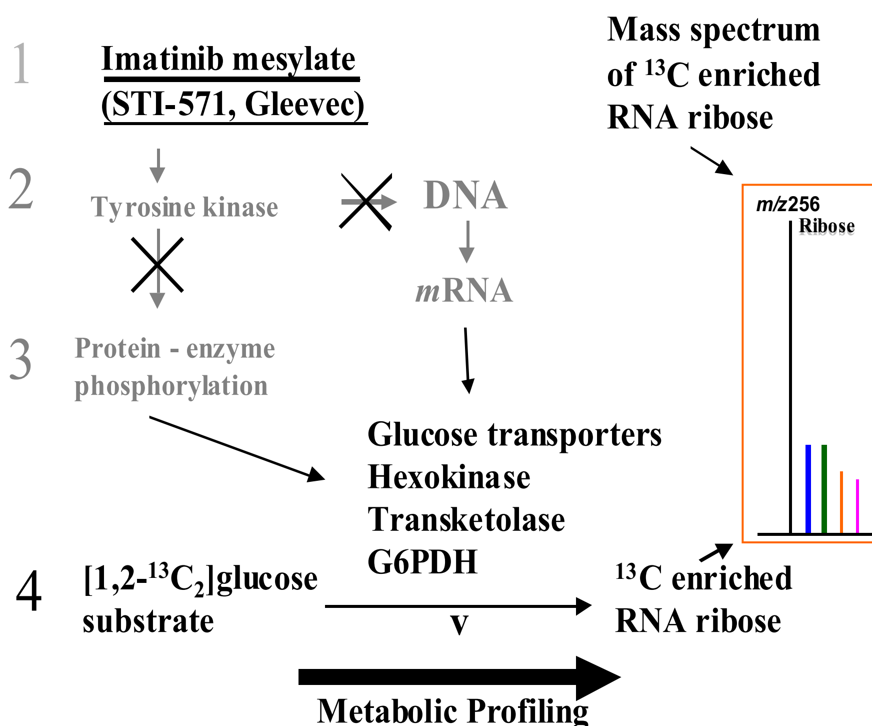

Fig. (4). Mass spectrum of ribose isolated from kinase inhibitor imatinib mesylate (STI-571, Gleevec) treated leukemia (K562) cells as revealed by metabolic profiling. The anti-leukemia agent imatinib mesylate effectively controls glucose carbon flow toward nucleic acid ribose synthesis in myeloid cells, resulting in cell cycle arrest and apoptosis. Metabolic profile changes include a decrease of ^{13}C accumulation into RNA, due to limited oxidative and non-oxidative ribose synthesis. The metabolic profile not shown here indicates that there is a significant increase in direct glucose oxidation and recycling in the pentose cycle as shown by increased m1/m2 ratio in lactate. This provides more reducing equivalents for cell differentiation through increased glycolytic substrate flow and decreased nucleic acid ribose synthesis, which limits cell proliferation. The metabolic profile shown here is also common in other anti-cancer treatment modalities (original data is reported in reference [48]). Thus the pattern of ribose labeling can serve as the substrate flow metabolic biomarker profile of effective cancer treatment modalities for experimental treatments to come.

transformation of myeloid cells in chronic myeloid leukemia (CML) [47]. Imatinib mesylate (STI-571, Gleevec), a novel BCR-ABL kinase inhibitor, effectively controls pentose cycle carbon flow by inhibiting metabolic enzymes, which results in limited glucose uptake, phosphorylation and utilization toward the synthesis of nucleic acid precursor ribose via the oxidative and non-oxidative branches of the pentose cycle [48] (Fig. **4**). This effect of imatinib is dose dependent and unique to the direct targeting of the kinase activity of BCR-ABL, as negative control cells did not exhibit a similar metabolic profile. Metabolic enzyme activity changes are also opposite to those induced by hydroxyurea, a potent DNA/RNA synthesis inhibitor in CML indicating a specific role of BCR-ABL and imatinib in pentose cycle carbon flow regulation, ribose synthesis and cell proliferation. At doses comparable to those used in the treatment of myelogenous leukemia, imatinib has also been shown to suppress hexokinase, glucose-6-P dehydrogenase (G6PD) and transketolase activities in K562 myeloid leukemia cells as the target metabolic enzymes of the blocked signaling cascade [48].

Similar substrate flow modifications, namely decreased glucose carbon flow toward nucleic acid synthesis have also been observed in cancer cells after treatment with other agents including tyrosine kinase inhibitor phytochemicals such as genistein [49] and the fermented wheat germ extract Avemar [50]. The fermented wheat germ extract effectively inhibits non-oxidative pentose cycle enzymes in a dose dependent manner and induces apoptosis in leukemia cells of the lymphoid lineage [51]. The mechanism involves glycolysis/pentose cycle enzyme targets and the induction of apoptosis through poly(ADP-ribose) polymerase activation. The tumor cell growth controlling effect of fermented wheat germ is uniformly effective in many human malignancies including pancreatic, colorectal, breast, lung cancers as well as leukemias, indicating that Avemar action does not depend on the presence of specific signal transduction pathways or oncogenes and that it attacks multiple tumor specific targets in tumor cells.

Direct inhibitors of the direct glucose oxidation pentose cycle pathway, such as dehydroepiandrosterone sulfate (DHEA-S) and the direct transketolase non-oxidative enzyme inhibitor oxythiamine inhibit tumor cell growth through cycle arrest and apoptosis similar to that observed in response to the kinase cascade signal inhibitor imatinib mesylate [52, 53]. Specific pentose synthesis patterns in tumor cells predict kinase effects as well as drug efficacy in experimental tumor growth. Therefore, cell growth limiting metabolic profiles can in the future be used to screen and test anti-cancer tyrosine kinase inhibitor treatment modalities for effectiveness and to identify mechanisms of anti-proliferative action throughout the pharmaceutical and academic drug development processes.

METABOLIC CONTROL ANALYSIS IN BIOMARKER AND DRUG DISCOVERY

In order to understand why pentose cycle substrate flow controlling enzymes (G6PDH and transketolase) have such a strong influence on cell physiology and how they control cell growth and transformation, one needs to consider the concept of Metabolic Control Analysis (MCA). MCA provides a quantitative description of how changes in system properties, such as metabolic fluxes or cell growth, are related with changes in system components such as gene expression or enzyme activities [54]. MCA and metabolic profiling are closely related fields as substrate flow changes are the combined results of gene expression, enzyme protein synthesis, enzyme activity changes and substrate availability and redistribution. These factors constitute integrated regulatory mechanisms of cell function, and they can only be understood as integrated elements of a cell's complex genetic, signaling and metabolic architecture. For example, the *in vivo* growth-control coefficient of transketolase in the *Ehrlich*'s tumor model was recently reported to be the highest (0.8) of all oxidative and non-oxidative pentose cycle enzymes [55] and this enzyme is now ranked as a new promising specific proteomics biomarker for cancer as well as a high efficacy anticancer drug target [56]. For example, tyrosine kinase altering drugs with significant inhibitory effects on glucose uptake or non-oxidative pentose cycle reactions are expected to exert potent and selective anti-cancer activities based on the strong control properties of transketolase in tumor cell nucleic acid synthesis as demonstrated above.

In other words, enzyme biomarkers and targets for new anti-cancer therapies have to be those that demonstrate strong control properties over glucose uptake, substrate flow for nucleic acid ribose synthesis, lactate production, or glucose anaplerosis in the TCA cycle. SIDMAP is crucial to identifying and characterizing new metabolic target sites for anti-cancer therapies to come. This is, of course, a crucial criterion in drug development efforts in which the efficacy of potential anti-proliferative drugs is determined by a metabolic screen in correlation with genetics and proteomics data.

METABOLITE PROFILING IN BIOMARKER AND KINASE BASED DRUG TARGET DISCOVERY: THE BENEFITS FOR INDUSTRY AND ACADEMIA

Metabolite profiling can identify new biomarkers that indicate the oncogenic disruption of unique metabolic networks in tumor cells versus the activation of others involved in macromolecule synthesis triggered by the activation of kinase based signaling cascades. However, specific biomarkers and target sites can only be identified by "smart" and comprehensive labeling of the metabolome, very much alike labeling DNA or proteins for genetic and proteomics studies. A combined study of signaling, genetic and metabolic events allows us to better define metabolic processes in cancer cell growth and death during drug treatment [57, 58]. One can expect the new stable isotope tracer-based metabolic profiling (SIDMAP) technologies to provide, in the near future, new biomarkers for cancer drug testing that reach beyond the known genetic and signaling patterns typically observed now. Characterization of the new metabolic biomarkers will supplement current testing methods and facilitate discovery of new signal driven intracellular mechanisms, genetic function and their modulations by new drugs.

Glucose substrate flux control is an important and common mechanism of anti-proliferative action by various novel anti-cancer drugs, downstream from their genetic and signaling effects. Although molecular genetic studies can anticipate changes in metabolism, they can not fully reveal whether metabolic enzymes are affected by kinase based signaling cascades or whether their substrates are relatively abundant or absent in differing, specific microenvironments. Therefore, metabolic profiling using stable isotope labeled substrates is needed for more complete understanding of "genotype-phenotype correlations". Because the metabolome represents critical events of phenotype modifications in substrate flow in cancer, metabolic profiling provides vital information regarding cell transformation and phenotypic modification beyond the scope of commonly applied signal transduction and genetic studies. Unlike genetic and proteomics studies, SIDMAP is also a quantitative tool to determine the level of cell transformation as well as cellular response to kinase inhibitor drug treatments based on quantitative changes in metabolite levels, substrate flux and macromolecule synthesis pathway activity ratios. Other advantages arise from the fact that stable isotope-based metabolic profiling does not interfere with current *in vitro* and *in vivo* experimental protocols because of the lack of biological effects of low enriched stable isotope labeled substrates and their intermediates in culture media or circulating blood. Therefore, metabolic profiling, genetic and proteomics studies can easily be performed in the same cultures or animals after incorporating the appropriate tracer substrate into the experimental design.

The application of SIDMAP also provides a new business model for the pharmaceutical industry by facilitating more comprehensive and mechanism-based evaluation of drug selection and drug testing procedures. Stable isotope labeling technologies can reduce the need for trial-and-error approaches in new drug development because a fuller understanding of drug effects and phenotypic modifications should be achieved early during the developmental processes. That earlier, fuller understanding can significantly increase the pace and the likelihood of drug approval while offering opportunities to also improve both the labeling and marketing of new drugs. By combining advanced mass spectrometry and “smart” glucose stable isotope tracers, tumors will soon be diagnosed by their unique metabolic activities imprinted in the serum metabolome.

ACKNOWLEDGEMENTS

This work was supported by the PHS M01-RR00425 of the General Clinical Research Unit and by P01-CA42710 of the UCLA Clinical Nutrition Research Unit Stable Isotope Core. The text of this article was co-edited by Dale Chenoweth of Austin, Texas.

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