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Cancer

Use of metabolic pathway flux information in targeted cancer drug design

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Knowledge of tumor-specific metabolic pathway flux, obtainable via the emerging tool of stable isotope-based dynamic metabolic profiling (SIDMAP), identifies metabolic enzyme drug targets which might help to overcome difficulties related to resistance to currently evolving targeted therapies against more narrowly conceived gene or protein targets. An accurate metabolic map detailing metabolic pathway substrate flow (flux) of the many tumor cell phenotypes is essential to design effective targeted cancer drugs and to accurately predict drug response.

Introduction

Tumor cells inherently possess various mechanisms to initiate and sustain any one of the following phenotypes: (1) proliferative; (2) differentiated; (3) transformed; (4) cycle arrested; (5) necrotic; and (6) apoptotic [1]. Advanced and therapy-resistant tumors share the characteristics of rapid proliferation, poor differentiation and resistance to apoptosis. They also exhibit a high rate of metabolism, using glucose as the primary substrate [2–5]. Therefore, factors that sustain tumor cells' sensitivity to exogenous and endogenous growth signals also reside deep within the METABOLIC NETWORK (see Glossary) supplying essential substrates for *de novo* macromolecule synthesis and energy production. The rate of pro-

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The high rate of metabolism of certain cancers affords an opportunity to understand better the pathways on which cancer cells might be dependent. Here, László Boros *et al.* review how metabolic profiling (e.g. glucose use and fatty acid synthesis) can clarify the mechanisms by which certain tumors develop resistance to molecular targeted therapies (MTTs). Ultimately, the use of such information could lead to the identification of new therapies that, when used in combination with MTTs, might offer new options in the treatment of cancer.

liferation is closely associated with the rate of *de novo* synthesis of macromolecules such as RNA, DNA, amino acids and fatty acids [6]. These complex molecules, which eventually become structural components of progenies of tumor cells, are synthesized from small molecular weight substrates, such as glucose, short-chain fatty acids and amino acids in an interconnected and complex metabolic network. All pathways in the network interact with one another via substrate sharing and channeling, and by regenerating shared co-factors that participate in oxidative degradation and reductive synthesis simultaneously. Such an interdependent relationship is evident between direct glucose oxidation in the pentose cycle and *de novo* fatty acid synthesis, where part of the reduced nicotinamide adenine dinucleotide phosphate (NADP⁺) pool is regenerated, allowing the irreversible glucose-6-phosphate dehydrogenase (G6PDH) reaction to proceed for the synthesis of five-carbon sugars [7,8]. In turn, a

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Glossary

Drug resistance: the drug loses effect against the target and thus the disease.

Isotopomer: a molecule which has one or more atomic substitution(s) with a heavy stable isotope tracer and the position(s) of substitution(s) are known.

Mass spectrometry: technology capable of determining and measuring the number and positions of heavy tracer atom substitutions in a biomolecule.

Metabolic network: where substrates are processed to yield energy or various macromolecules that a cell needs to survive and function in an organ or host.

Targeted drug design: the design of a drug against a well-defined and characterized molecular target.

vast amount of the reducing NADP⁺ equivalent is used during reductive *de novo* synthesis of fatty acids, their chain elongation and de-saturation, allowing distant metabolic reactions of the same network to proceed in a well-controlled and synchronized fashion. Cell phenotypes and their sensitivity to apoptosis, on which this review focuses, demonstrate differences in their respective stable isotope-labeled dynamic metabolic profiles (SIDMAPs) of cross-regulated metabolic pathways in the network. The models discussed herein include therapy-resistant inflammatory breast cancer cells in comparison with apoptosis-sensitive human fibroblasts and therapy-sensitive pancreatic tumor cells. Unlike traditional chemotherapy, new TARGETED DRUGS (see Glossary) accurately mend abnormal functions of single genes and proteins, as well as affect a narrow range of metabolic downstream reactions. Yet, because metabolic networks inherently possess wide functional flexibility because of the existence of multiple alternate macromolecule synthesis pathways, which lack control by a given target gene or protein, targeted drugs can fail to control a broad range of tumor cell phenotypes and allow eventual development of DRUG RESISTANCE (see Glossary and Outstanding issues). In this paper, we argue that it is possible for targeted therapies of the metabolic network to overcome limitations of drug design related to the enormous variability of the ever-changing pool of genetic and proteomic targets in cancer [9–12]. However, one has to learn first how to trace the pathways taken by precursors of nucleic acids and cell structure components as they become constituent components of macromolecules. Subsequent informed reading of such a map, tracing the dynamic history of metabolic reactions resulting in macromolecule synthesis identifies multiple opportunities to block progress along those interconnected pathways.

Targeted cancer therapies aimed at genetic and proteomic targets

Targeted drugs are most commonly designed to focus on a single or a very narrow range of genetic or protein targets.

Although they are extremely effective to treat narrow cancer cell populations, and typically have very limited toxicity to the host, their main limitations are high dependence on a single highly modifiable target and the rapid development of resistance based on four major mechanisms: (1) decrease in target protein expression; (2) mutations in target proteins; (3) loss of target gene and/or construct owing to clonal selection; and (4) increased drug transport from targeted cells. These limitations have produced significant delays, cost accumulations or disappointments with almost all targeted drugs designed so far shown in Table 1.

It is evident that although the targeting of narrow oncogenic constructs with “magic bullets” is an old approach that raised high expectations, multiple mechanisms slipping the target continuously off center in the ever-changing genetic and proteomic maze of targets make this approach ever more expensive. One solution is to re-design drugs with altered structure to hit a new, wider range of targets [10], but this approach is controversial, given the difficulties and costs involved in repeated clinical trials for each new drug targeting slightly mutated proteins. This approach will probably exhaust research and development budgets of even large drug companies in the long run, because the cost of targeted therapy drug design is already enormous compared to conventional treatments, whereas the return is limited owing to the relatively small numbers of patients who receive benefit from them. It is also evident that health insurance companies will not be able to cover the ever-growing costs of targeted therapies administered for as long as it would be necessary to keep the ever-changing genetic and protein profiles driving tumor growth under control [13]. Considering even just a few of the possible meaningful oncogenic permutations that can be expected from gene and protein variations in an evolving population of malignant cells, targeted therapies against genetic and proteomic network targets might well be the most expensive journey medicine has yet taken to yield so little aggregate improvement in cancer outcomes.

Metabolomics: the study of the transformed metabolic network in cancer

Metabolomics is a new field in medicine addressing and developing enhanced disease understanding platforms with the potential to design significantly more effective drugs against many human diseases, including cancer [14]. Metabolomics is a tool designed around quantitative measurement of metabolite levels and ratios, which are obtained and mined using several pattern recognition techniques, including principal components analyses and their hierarchical clusters. This combined approach is termed metabolomics, and foresights into the field predict strong roles in medicine that are both complementary to and competitive

Table 1. Targeted therapies of cancer and factors responsible for failures

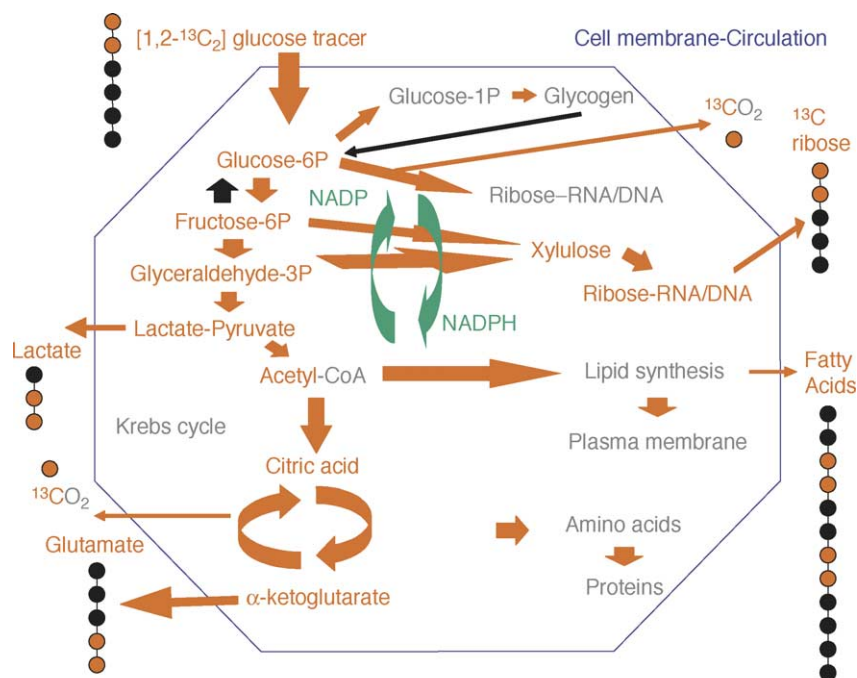
	Pros	Cons	Latest developments	Who is working on this strategy	References
Monoclonal antibodies, EGF mABs and small molecule receptor antagonists	High specificity against cytokines and cell surface receptors	Host immune response, increased cytokine production	Low response rate, especially in refractory disease	AstraZeneca; www.astrazeneca.com	[32]
Conjugated toxins or radioisotopes in leukemia	Targeted delivery, high efficacy drugs	Transporter and receptor dependent	Low response rate and recurrence	Fred Hutchinson Cancer Research Center	[33,34]
Antisense oligonucleotides (ISIS 5132; CGP 69846A)	Gene expression modifying RNA-specific oligonucleotides	Delivery system is still not resolved, severe host response to viral vectors	Hemolytic anemia, renal failure and anasarca	Isis Pharmaceuticals; www.isis.com	[35]
Immunoliposome-encapsulated drugs	Targeted delivery of protected antibodies	Moderate stability, inability of the carrier to extravasate ^a	Low efficacy, few clinical trials in progress and high failure rate	Institute of Radiology and Pathophysiology, German Cancer Research Center	[36]
Small molecule inhibitors	Targets single oncogenic protein construct	Recurrence with blast disease in CML	Drug resistance is an emerging and severe problem	Novartis; www.novartis.com	[37]
Imatinib					

EGF, epidermal growth factor; mABs, monoclonal antibodies; CML, chronic myeloid leukemia.

^aLeave the bloodstream to enter cells.

with genetics and proteomics. Although there is still important reliance on functional genomics to elucidate the roles of genes and their protein products in human cancer, there is also an increasing awareness of the necessity to define and understand in full detail how the genetic and protein networks interact with and are influenced by the metabolism of particular tumor phenotypes [15]. Although it is known that metabolic networks also vary in their substrate utilization patterns and flux distribution, their control points have been strictly preserved throughout evolution and are very reliable drug targets considering the limited number of enzyme *iso*-forms as well as the limited number of major alternative metabolic routes, which are mostly known (see Outstanding issues). Metabolism is an old field with new potential to assist drug development, once one understands how to navigate among the many interconnected pathways and how to define targets and trace reaction histories in the same network. Fig. 1 demonstrates the theoretical accumulation of the SIDMAP tracer into various intra-cellular and extra-cellular metabolites of a human cell. Just as other major fields of medicine already have greatly benefited from effective tracer technologies such as polymerase chain reaction in genomics/transcriptomics research and monoclonal antibodies used in proteomics-based research, stable isotope-based dynamic metabolic profiling will also enhance our understanding of metabolic network behavior in resistance to new targeted therapies in cancer. The main use of tracer-based metabolic flux information, also called fluxomics, is the recognition, dissecting and detailed analysis of alternative macromolecule synthesis pathways to develop new treatment strategies when drug resistance occurs. Revealing mechanisms of early adaptation of the metabolic network to currently effective drugs will help scientists to prepare and overcome complex mechanisms of drug resistance through a better understanding of the underlying mechanisms of flux changes downstream of the targeted genetic and proteomic mechanism(s) of drug response.

The double tracer approach, using the stable isotope ¹³C to label the first two carbon positions of the glucose molecule, is particularly effective in revealing detailed substrate flow and distribution patterns in the complex metabolic network of human cells. This approach is capable of providing detailed mapping of cellular substrate utilization, including destinies of intermediary metabolites. Such detailed metabolic information, not otherwise obtainable, is invaluable for drug target discovery and for measuring drug effects. Identification of the destinies of metabolites is achieved by determining the number and positions where tracer glucose carbon atoms reside in bio-molecules of interest. Tumor cells show transformed and altered metabolic profiles based on their sensitivity to growth signals and apoptosis as described below.



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Figure 1. Stable isotope-based dynamic metabolic profile (SIDMAP) of a mammalian cell. Red arrows indicate routes of ^{13}C tracer glucose substrate carbons (red filled circles) in the metabolic network. The heavy use of glucose carbons via intermediary metabolism and *de novo* macromolecule synthesis reactions allows detailed analysis of metabolic pathway substrate flow (flux) in disease and health.

Stable isotope labeled metabolic network and sensitivity to apoptosis: two models

*Apoptosis-sensitive cells depend heavily on non-oxidative pentose cycle metabolism although lacking *de novo* fatty acid synthesis*

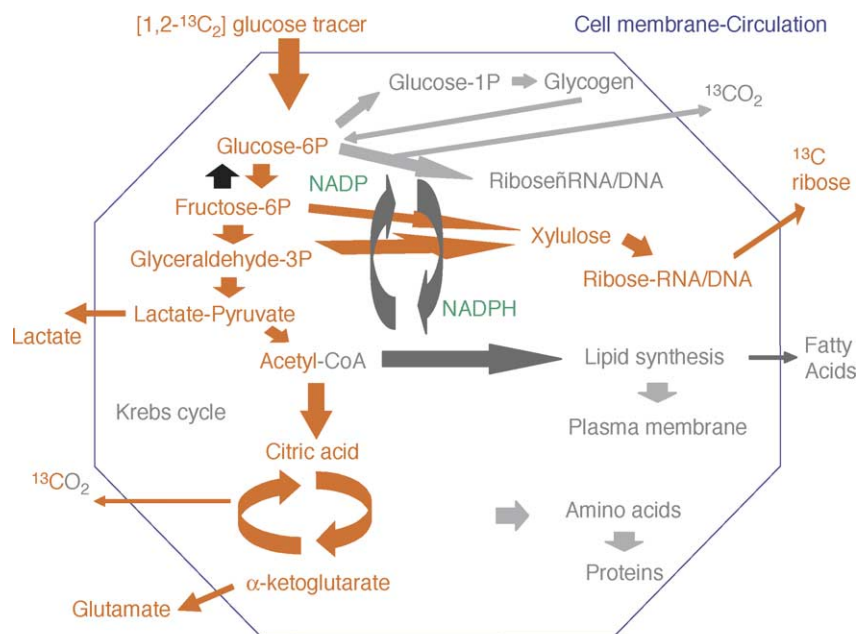
SIDMAP applications in cancer have provided greatly improved understanding of growth controlling mechanisms in the transformed metabolic network [16,17]. Stable isotope-based dynamic metabolic profiling (SIDMAP) has revealed that the inability to synthesize fatty acids through pentose cycle metabolism makes human fibroblasts extremely vulnerable to apoptosis when cell access to thiamine, a necessary co-factor of the transketolase non-oxidative pentose cycle enzyme, becomes limited owing to defective high-affinity thiamine transport [18,19]. Thiamine transport-deficient human fibroblasts readily undergo apoptosis in culture with no rescue mechanism in place because their inability to synthesize fatty acids *de novo* severely limits their reserves of the oxidized form of NADP^+ , which is the sole hydrogen acceptor during oxidative pentose synthesis from glucose in the pentose cycle. Similarly, pancreatic adenocarcinoma cells (MIA PaCa) show limited growth when treated with pentose cycle inhibitors [20] and possess a relatively low rate (20%) of *de novo* fatty acid synthesis and turnover during the 72 h treatment period [21,22]. Fig. 2 demonstrates metabolic pathway substrate flow in apoptosis-sensitive human cells using flux array information originating from the accumula-

tion of ^{13}C tracer in intermediary metabolites and bio-molecules.

*Apoptosis-resistant cells depend heavily on oxidative pentose cycle metabolism, maintaining high rates of *de novo* fatty acid synthesis and turnover*

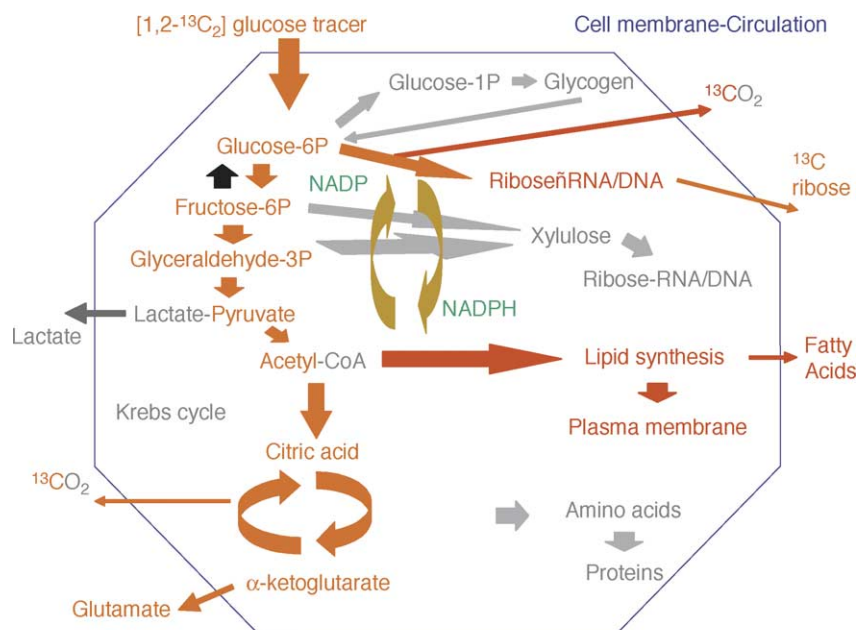
The SIDMAP metabolic profile of therapy-resistant and apoptosis-resistant tumor cells is different from that of therapy-sensitive cells shown in Fig. 2. Fig. 3 illustrates the metabolic profile of therapy-resistant inflammatory breast cancer cells, indicating intense tracer accumulation into fatty acids and oxidation of NADPH.

The main metabolic difference between the apoptosis-sensitive TRMA cells and the apoptosis-resistant IBC cells is the relatively much more rapid rate at which the IBC cells synthesize medium and long chain saturated fatty acids up to the 16 carbon chain length palmitate and consequently elongate it to the 18 carbon length stearate and further into C:20–C:26 species. The IBC cells also possess high fatty acid chain desaturase activity, further oxidizing NADPH and allowing the oxidative branch of the pentose cycle to operate under drug treatment. This is especially important when nucleic acid synthesis inhibitors are targeting either branch of the pentose cycle, because operation of at least one of these alternative synthesis routes is essential for significant portions of tumor cell populations to survive and replicate, while



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Figure 2. Stable isotope-based dynamic metabolic profile (SIDMAP) of *apoptosis sensitive* human cells. Flux array is shown by color codes known from SIDMAP array analyses (see later), where black and gray colors indicate limited flux, whereas yellow and red indicate increased flux. The non-oxidative route is heavily used in the pentose cycle of human fibroblasts (TRMA cells), whereas the oxidative pathway is limited owing to low NADP-NADPH cycling and fatty acid synthesis. Human fibroblasts with high-affinity thiamine transport deficiency readily undergo apoptosis [18] and MIA pancreatic adenocarcinoma cells show slowed growth in response to non-oxidative pentose cycle inhibitors [20].



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Figure 3. Stable isotope-based dynamic metabolic profile (SIDMAP) of *apoptosis-resistant* tumor cells. Red arrows indicate routes of ^{13}C tracer glucose substrate carbons (red filled circles) in the metabolic network. NADPH-NADP cycling is active and is compensating in response to non-oxidative pentose cycle inhibitor treatment. Inflammatory breast cancer (IBC) cells exhibiting this SIDMAP are extremely durable, treatment resistant and aggressive. Although growth retardation is achieved, IBC cells can not be forced into apoptosis even when the toxic glucose derivative 2-deoxy-D-glucose is given in high doses (5 mM) [23].

enduring apoptosis-inducing drugs and signals, and thereby for tumors to survive.

Metabolic profile of Gleevec (STI-571) resistance in chronic myeloid leukemia

The gene product resulting from the breakpoint cluster region – Abelson (BCR-ABL) chromosomal fusion causes metabolic profile changes to occur and to remain constitutively expressed in BCR-ABL-positive (BCR-ABL⁺) leukemia cells. Hematopoietic cells transfected with BCR-ABL express the high-affinity glucose transporter (GLUT1) and increase their glucose uptake as the metabolic hallmark of their transformation (see Related articles) [24]. This transformation also involves the activation of subsequent glucose-metabolizing enzymes, hexokinase-II and glucose-6-phosphate dehydrogenase [25]. Imatinib mesylate (Gleevec) effectively controls these enzymes, which results in limitation of glucose uptake, glucose activation (phosphorylation) and direct oxidative decarboxylation toward the synthesis of nucleic acid precursor ribose as well as NADP⁺

via the oxidative branch of the pentose cycle (see Related articles) [26]. Although imatinib also reverses the Warburg effect in BCR-ABL-positive cells by switching from glycolysis to mitochondrial glucose metabolism, which results in decreased glucose uptake and higher energy state [27], it does not control the non-oxidative reactions of the pentose cycle, which are alternative pathways of nucleic acid RNA and DNA macromolecule synthesis. Although an effective control on direct glucose oxidation, fatty acid synthesis and TCA cycle metabolism is achieved by Gleevec in therapy responsive cells, there is, unfortunately, also a dose-dependent increase in non-oxidative RNA ribose synthesis as documented four years ago [26]. Activity of transketolase, which is the rate limiting enzyme of non-oxidative ribose synthesis [28,29] is only imperceptibly inhibited by Gleevec, giving rise to a metabolic phenotype of non-oxidative ribose synthesis, and decreased glucose oxidation in the pentose cycle and fatty acid synthesis (Fig. 4).

The mechanisms of resistance to Gleevec of K562R and LAMA84R cells shown in the array are different, yet their

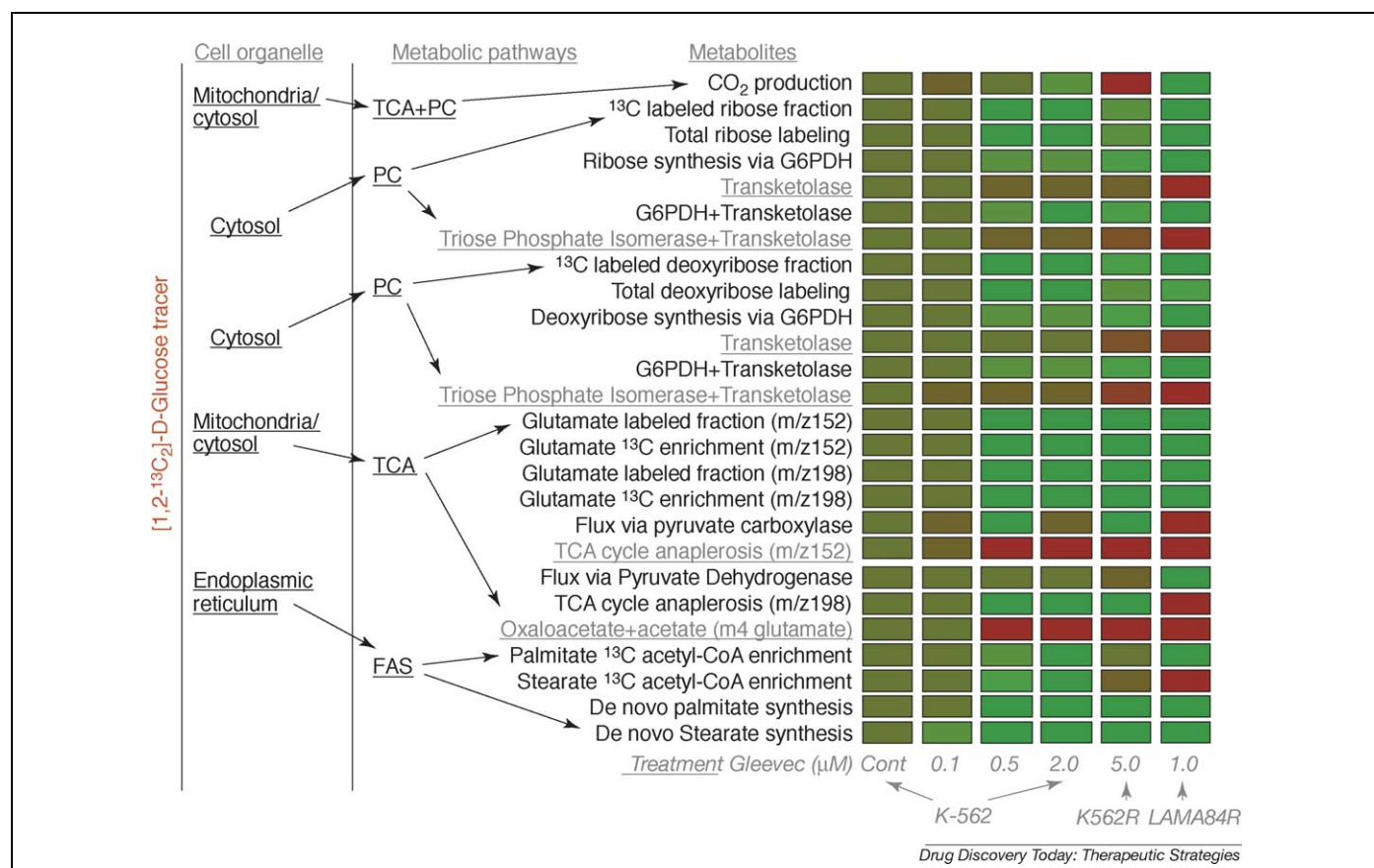


Figure 4. Stable isotope-based dynamic metabolic profile array (SIDMAParray™) of Gleevec sensitive (K-562) and two Gleevec resistant tumor cell lines (K-562R and LAMA84R). Red spots indicate routes of ¹³C tracer glucose substrate carbon incorporation into various molecules as per cent of flux in control untreated cells produced in the pentose cycle (PC) and tricarboxylic acid (TCA) cycle. Metabolic pathways, which lack control by Gleevec in sensitive and resistant cells are underlined and yellow in the array. These include non-oxidative ribose/deoxyribose synthesis reactions via triose phosphate isomerase and transketolase. Metabolic pathways rigorously controlled by Gleevec, including the oxidative decarboxylation of glucose in the pentose cycle and de novo palmitate and stearate synthesis, are indicated by green spots. An interactive SIDMAParray™ is available at www.sidmap.com (FAS: fatty acid synthase; G6PDH: glucose 6-phosphate dehydrogenase).

metabolic phenotypes of lacking control of non-oxidative ribose/deoxyribose synthesis by Gleevec are similar.

K-562R cells are resistant to 5.0 μ M Gleevec (results are from Dr Moshe Talpaz's laboratory at MD Anderson Cancer Center, http://www.mdanderson.org/cancer_pro) and they show:

- Reduced BCR-ABL expression and limited activation of BCR-ABL signaling cascades (Stat 5, CrkL, MAPK) – BCR-ABL not coupled to proliferation and survival of K562R cells [30].
- Src related kinase LYN highly overexpressed and activated [30].
- No pgp (p-glycoprotein) overexpression.

LAMA84R cells, resistant to 1.0 μ M Gleevec (results are from Dr Junia Melo's laboratory, Department of Haematology, Imperial College School of Science, Technology and Medicine, Hammersmith Hospital, London, UK; http://www.med.ic.ac.uk/divisions/template_divisions_departments.asp?id=10), show:

- Up-regulation of BCR-ABL protein associated with amplification of the BCR-ABL gene [31].
- pgp overexpression [31].

Applications of tracer-based metabolic flux information to detect Gleevec resistant cells

As stable isotope tracer studies can readily be performed in experimental animals or humans with no toxicities, it is possible to profile peripheral blood cells in the presence of Gleevec treatment. The effect of Gleevec therapy and early signs of its failure can potentially be predicted from the metabolic profile of leukemia cells by the increase of non-oxidative metabolite synthesis as shown in the SIDMAParray™. New therapeutic strategies can also be elaborated using metabolic flux information identifying enzymes which lack or escape control, both in cells that are Gleevec-sensitive and those that are resistant to the drug.

Future metabolic drug design scenarios based on what we know already

Genomics and proteomics studies have generated enough data in the past two decades that now targeted therapies against unique genes and proteins are used in clinical practice for the treatment of cancer. However, significant resistance to new targeted therapies occurred rather quickly and frequently, bringing new challenges back to the laboratory. As metabolic tracer data increasingly accumulates, revealing highly significant but previously unknown operational details of the transformed metabolic network of tumor cells, new opportunities have arisen to force potentially malignant cells into apoptosis through design of therapies focused on key metabolic targets essential to tumor cell survival and

replication. The current challenges are several. First of all, more SIDMAP data are needed and they must be correlated with tumor phenotypes, biological behavior and metabolic network characteristics. Secondly, it is probable that therapies targeted against tumor metabolism will have to be aimed at multiple sites in the network owing to the fact that metabolic networks are interconnected and alternative synthesis pathways are common. Tumor SIDMAPs can easily be determined both *in vitro* and *in vivo*, using non-invasive, non-radiating, natural sugar tracers for both diagnostic and metabolic targeting purposes.

Conclusions

Currently there are significant limitations common to the design and function of molecular targeted drugs because, unlike more traditional chemotherapies, molecular target drugs only control metabolic pathways downstream of the targeted gene or signaling construct. Alternative macromolecule synthesis pathways are evident in drug-sensitive cells and might become highly active in targeted, therapy-resistant cells. It is crucially important to know exactly what it is that the specific, targeted genes and signaling constructs *do* and *do not* control within the metabolic network. Inhibitors of metabolic enzymes involved in drug resistance, revealed by characterizing resistant metabolic phenotypes, will in the future probably be key compounds to complement the narrow range of actions that targeted therapies have in the metabolome. It is now possible, applying the SIDMAP approach, to develop strategies to preclude development of drug resistance before it appears, and to pinpoint disease mechanisms in the metabolome that result from mutated, dysfunctional proteins and their downstream effects in the metabolic network (see Related articles). Stable isotope-based dynamic metabolic profiling provides the opportunity to conduct “clinical trials in a test tube”, identifying efficacy limitations and unexpected side effects of narrowly conceived targeted drugs before the drugs are given to humans or are marketed. Such use of newly available metabolomics tools can preclude the significant patient harm and enormous market-share losses, too often seen when a drug company is forced to limit sales or withdraw a drug owing to unforeseen limitations of targeted drug design, which could have been revealed by advanced tracer technologies applied to the metabolome in a very early phase of the drug's development.

As metabolic profiling reveals new targets, the promise of these new classes of metabolomic targets is that they have no ability to escape drug inhibition by mutations, as do genetic and signal protein targets. This is because structures of metabolic enzymes and hierarchies of metabolic networks are well preserved throughout evolution, and tumor cells still have to adhere to the obligatory biochemical requirements of this limited number of hierarchies to survive. Regardless of the

Related links

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level of transformation and malignancy, tumor cells have to integrate their metabolism with that of a complex host operating on a limited number of substrates and co-operative futile cycles. It is evident that mutations in growth signal proteins effectively shield them from drugs meant to target them, yet commonly, the pathologic functions of the protein are not lost or lessened, and might even in some instances be increased. Many growth signals exist contemporarily, initiating downstream effects that can become constitutively active and they can maintain signaling even through multiple, sequential variations in gene expression. By contrast, although mutations in metabolic enzyme proteins might also allow them to escape newly designed drugs meant to target them, any such mutation(s) also typically make the metabolic enzyme defective and unable to catalyze the metabolic reaction, which the inhibitor is meant to control. The purpose of applied metabolomics tools such as SIDMAP in the new, targeted era of cancer drug design is to pinpoint metabolic network targets essential to pathologic processes and malignant cell survival in the fundamental component of cell function, the metabolome. These targets have very limited ability to develop effective resistance by point mutations or structural/conformational changes, or any of the other mechanisms that make genetic and protein targets weak and short-lived. For these reasons, a focus on targets selected carefully from the increasing metabolomic data now becoming available is probable to overcome the problems of drug resistance and limited applicability that currently plague targeted cancer therapies, leading to more sustained responses and better patient outcomes.

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Related articles

- Bentley, J. *et al.* (2001) Glucose transport regulation by p210 Bcr-Abl in a chronic myeloid leukemia model. *Br. J. Haematol.* 112, 212–215
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manuscript received its final edit from Dale Chenoweth, of Austin, TX, USA.

Outstanding issues

- Are there more than a few metabolic profiles of tumors?
- Is there a way of designing targeted drugs against multiple reactions in a metabolic network?
- How are metabolic enzyme drug targets different from molecular signaling proteins with regard to mutations and to escaping drug effects?

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