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Use of nuclear magnetic resonance-based metabolomics in detecting drug resistance in cancer

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Abstract

Cancer cells possess a highly unique metabolic phenotype, which is characterized by high glucose uptake, increased glycolytic activity, decreased mitochondrial activity, low bioenergetic and increased phospholipid turnover. These metabolic hallmarks can be readily assessed by metabolic technologies – either *in vitro* or *in vivo* – to monitor responsiveness and resistance to novel targeted drugs, where specific inhibition of cell proliferation (cytostatic effect) occurs rather than direct induction of cell death (cytotoxicity). Using modern analytical technologies in combination with statistical approaches, ‘metabolomics’, a global metabolic profile on patient samples can be established and validated for responders and nonresponders, providing additional metabolic end points. Discovered metabolic end points should be translated into noninvasive metabolic imaging protocols.

Keywords

anticancer treatment; cancer biomarkers; choline metabolism; quantitative metabolomics; signal transduction inhibitor; Warburg effect

The development of novel anticancer treatment regimens requires both the precise identification of the patient population that will most likely respond to specific targeted therapy and reliable surrogate markers to follow-up treatment efficacy. The concept of a biomarker can encompass anything from a single molecular species (gene, protein, metabolite) to a complex fingerprint of molecular changes (genomics, proteomics, metabolomics) indicative of human pathology, for example, cancer [1–4]. Biomarkers are quantitative measurements of biologic homeostasis that can (specifically and sensitively) distinguish between ‘normal’ and ‘abnormal’. Ideally, their changes will be highly specific to therapeutic intervention, such as discriminating between ‘responders’ and ‘nonresponders’. Existing biomarkers include: radiological (tumor size and metastasis detection), genomic (Philadelphia chromosome), proteomic (Bcr-Abl, EGF receptor [EGFR] or CA125) or metabolic parameters (glucose uptake, choline or citrate spectroscopic images). Radiological end points, by MRI or computed tomography (CT) remain a gold standard for tumor detection and general assessment of

treatment efficacy. Gene and protein biomarkers (using gene arrays or conventional northern/western blots) can be superior for initial patient selection to the specific targeted drug regimen, while metabolic markers by noninvasive magnetic resonance spectroscopy/ spectroscopical imaging (MRS/MRSI) or PET are highly promising in following the efficacy of targeted treatment (FIGURE 1). Thus, one of the most important advantages of nuclear magnetic resonance (NMR)-based metabolomics is that after initial (invasive or semi-invasive) tissue analysis using high-resolution NMR spectrometers, the distinguished metabolic markers can be followed noninvasively in the body using clinical MR scanners, by collecting *in vivo* MRS (spectra) or MRSI (metabolite imaging) in the tissue. The selected metabolite must, however, be highly abundant in the tissue of interest in order to be visible by MRS and/or MRSI.

Metabolomics, an 'omic' science in systems biology, is the global quantitative assessment of endogenous metabolites within a biologic system. Metabolomics is a term that encompasses several types of analyses, including:

- Metabolic fingerprinting, which measures a subset of the whole profile followed by multivariate spectral analysis (principal component analysis, partial least square discriminant analysis) with little differentiation or quantitation of metabolites;
- Metabolic profiling, the quantitative study of a group of metabolites, known or unknown, within or associated with a particular metabolic pathway;
- Target isotope-based analysis, which focuses on a particular segment of the metabolome by analyzing only a few selected metabolites that comprise a specific biochemical pathway [2–13].

Interestingly, in cancer (unlike in toxicology), only a limited number of metabolomics studies [8,9] have utilized pattern recognition techniques on spectral data sets without identifying precise metabolic biomarkers of a specific cancer type. Most of the current metabolic studies are conducted in a mechanistic pathway-discovering manner, where identified and quantified metabolic markers (either as single metabolites or as metabolic pathways) provide precise insight into cancer biology [10–13]. This may be partly due to the compromised history of cancer metabolomics, dating back to the reported scientific misconduct of using a nontargeted multivariate NMR approach on the blood of cancer patients [14,15]. Another factor in support of precise metabolite identification and validation is the translational application of *in vivo* metabolomics or metabolic imaging (FIGURE 1).

Toxicology remains the major focus for NMR-based metabolomics of body fluids. Reported metabolic markers of nephro- and hepato-toxicity (two major target organs of drug toxicity) can be easily assessed in urine and/or blood owing to the crucial role of kidney and liver in performing global body metabolism. Unfortunately, the situation in oncology is much more complex. Solid tumors, even when quite large, have less impact on total body metabolism than kidney or liver dysfunction. The presence of circulating tumor cells in the blood is also not adequate to disturb metabolic homeostasis (since sensitivity of NMR-based metabolomics in detection of metabolic abnormalities is relatively low). Therefore, the most predictable way to assess metabolic abnormalities in tumor progression and responsiveness to anticancer therapies still relies on metabolic assessment of the tumor tissue itself. However, performing biopsies of solid tumors in patients on a routine basis to assess responsiveness to therapy is obviously not feasible. Hence, *in vitro*-discovered tumor tissue bio-markers need to be translated into noninvasive imaging protocols for routine assessment of treatment response. The comprehensive work of colleagues, both clinical and basic science, has demonstrated a clear proof of concept for *in vitro*-discovered, *in vivo*-validated biomarkers in translational metabolic profiling research [3,4,16,17]. As of today, two major metabolic pathways – namely glucose metabolism and phospholipid turnover – have been repeatedly reported to be involved in oncogenesis and their possible role in early prediction of therapy response is under

investigation using both *in vitro* metabolomics, and *in vivo* metabolic imaging [1–4,6,8,11, 13,16–23]. In addition to these general metabolic markers of malignancy, specific endogenous metabolites are implicated in particular tumors such as *N*-acetyl aspartate in neuroblastoma, *myo*-inositol in glioma, and citrate in prostate cancer, based on tissue-specific biochemistry.

This review describes existing NMR-based approaches for global metabolic profiling in tissue biopsies, body fluids and, finally, noninvasive assessment of metabolic biomarkers using noninvasive radiological techniques. The most recent studies on metabolic response and resistance development to novel targeted drugs (tyrosine kinase inhibitors [TKIs], metabolic modulators) are analyzed.

Metabolomics technologies

Either individually or grouped as a metabolomic profile, the detection of metabolites is usually carried out in cells, tissues or biofluids by either NMR spectroscopy or mass spectrometry (MS) [1–7]. In general, NMR spectroscopy, mostly ^1H -NMR, and MS, particularly liquid chromatography (LC)-MS and Fourier-transform ion cyclotron resonance-MS, are the two major spectroscopic techniques utilized in metabolic analysis. The basic workflow for NMR- as well as MS-based studies is as follows: quenching/extraction of metabolites → data collection → data processing/analysis [1,24]. NMR exploits the behavior of molecules when placed in a magnetic field, allowing the identification of different nuclei based on their resonant frequency when exposed to magnetic fields. MS determines the composition of particles based on the mass-to-charge ratio in charged particles. The resultant metabolite detection and quantification is acquired as a data set known as a spectrum. Each technique has distinct advantages and disadvantages [25]. The major advantages of ^1H -NMR include its non-biased metabolite detection, quantitative nature and reproducibility [1–4]. ^1H -NMR can be used for liquid or solid samples, using magic angle spinning (HR-MAS) techniques, with minimal sample preparation. ^{31}P -NMR of tissue specimens and cultured cells reflects products of energy or phospholipid metabolism, whereas ^{13}C -NMR measures dynamic carbon fluxes, such as glucose metabolism [6]. ^{13}C -NMR can be performed on tissues and cell extracts following incubation with a ^{13}C -labelled precursor [6,18]. Another significant advantage of NMR is that metabolic markers discovered and analyzed *in vitro* can be measured *in vivo*, presuming their high intra-tissue abundance, using another MR-based technology, namely localized MRSI (FIGURE 1).

Information on sample requirements and handling for metabolomics analysis has been published previously [1]. In brief, all biological samples collected for metabolic analysis require careful sample handling, such as special requirements for diet, physical activities and other patient validation prior to sample collection, as well as maintaining low temperature and consistent sample extraction afterwards (due to the high susceptibility of metabolic pathways to exogenous environment). For biofluids, the standard sample volume is accepted to be in the range of 0.1–0.5 cc. For NMR, minimal sample preparation is required for urine and other low-molecular-weight metabolite-containing fluids (TABLE 1). Blood, plasma and serum can yield more metabolic information if extraction (using acid, acetonitrile or two-phase methanol/chloroform protocols) or NMR-weighted techniques are applied to separate polar and lipophilic metabolites. Cancer cells and/or tissue biopsies usually undergo acidic extraction (7–12% perchloric acid, for example) or dual-phase methanol chloroform extraction. The extraction step and consequent lyophilization of the extract allows for:

- Separation of lipid- and water-soluble metabolites to prevent peak overlapping
- Precipitation of proteins and other macromolecules to avoid peak contamination and line broadening

- Obtaining liquid-state spectra from tissue specimens with high resolution and low-line broadening

Based on our experience, acidic extraction allows for better recovery of energy-rich phosphate metabolites, while dual-phase extraction has a better recovery rate for lipid-containing metabolites. Lyophilized extracts (body fluids, cells, tissues, cell culture media) are then usually redissolved in deuterated solvents and analyzed by conventional 5-mm high-resolution NMR probes (for ^1H -, ^{13}C - and ^{31}P -NMR, typically). The usual magnetic field strength for metabolic studies is between 7.0 and 14.1 Tesla (300–600 MHz for proton frequency). Some NMR-based cell metabolic studies (especially those using ^{13}C -fluxes by ^{13}C -NMR or ATP kinetics and pH calculation by ^{31}P -NMR) have used perfused cultured cells using large-diameter probes (10- to 20-cm instead of conventional 5-cm probes). Finally, intact tissue specimens (e.g., biopsies, fine needle aspirates) are increasingly used with the introduction of HR-MAS. HR-MAS probes for solid-state NMR, as well as cryoprobes and microprobes for liquid NMR, allow for quantitative NMR metabolic analysis on samples as small as 3 μl with significantly improved signal-to-noise ratios and solvent suppression. In general, there is no standardized protocol for sample preparation for NMR analysis, and the protocols presented here are based on literature reports and our experience in the field of NMR metabolomics in the past 15 years.

While NMR exploits the behavior of molecules when placed in a magnetic field, allowing the identification of different nuclei based on their resonant frequency, MS determines the composition of particles based on the mass:charge ratio in charged particles. The MS analysis requires more labor-intensive (and destructive) tissue preparation than NMR, but its advantage is higher sensitivity for metabolite detection. MS is highly sensitive, typically at the picogram level, and allows for specificity in metabolite identification at low concentrations and for more compounds to be screened when compared with NMR. While polar molecules may be detected when electrospray ionization is used, nonpolar molecules may require atmospheric pressure chemical ionization. Similarly, the methods of extraction, quenching, and sample storage conditions can affect and potentially modify metabolite structure, thereby confounding already complex data sets and introducing more sample-to-sample variability. Although high-resolution profiling methods exist for gas chromatography mass spectrometry profiling, detectable compounds are limited to those that can be derivatized, and derivatization can be time consuming and costly, and there is a risk of metabolite loss. Conversely, LC/MS has only recently begun to be applied to metabolic profiling owing to recent major advances in chromatography, instrumentation, ionization capabilities and software. In spite of a rich history of discovery- and targeted-based methods in small molecules using MS, a widely adopted and validated methodology for sensitive, high-throughput discovery-based LC/MS metabolomics is still lacking.

Cancer metabolic phenotype

Cancer represents an ideal application for metabolic profiling. The biochemistry of tumors, especially glucose and phospholipid metabolism, differs dramatically from normal cells. Since a Nobel Prize was nominated to Otto Warburg in 1931 [26,201], it has been widely known that cancer cells possess unique metabolic machinery to support their loss of cell cycle regulation. TABLE 2 shows major metabolic hallmarks of cancer cells, all of them related to high proliferation rate, mitochondrial dysfunction and increased glycolytic rate.

Warburg effect & glucose metabolism

The first identified biochemical abnormality of oncogenesis, identified by Otto Warburg in the 1920s [26,201], was a shift in glucose metabolism from oxidative phosphorylation to 'aerobic' glycolysis, meaning that tumors exhibit anaerobic metabolism (lactate production through

glycolysis) even in the presence of adequate oxygen [27–31]. In cancer cells, glucose is increasingly transported into the cell by glucose transporters (Glut-1 is most relevant for oncology owing to its overexpression in the majority of cancers and its application in fluorodeoxyglucose [FDG]-PET; Glut-3 over-expression has also been reported in a wide range of cancers) and undergoes glycolysis and the pentose phosphate pathway in the cytosolic fraction (FIGURE 2). Pyruvate, an intermediate of glycolysis, is then directly metabolized to lactate (the end product of glycolysis) by lactate dehydrogenase (LDH) or transported into the mitochondrion to enter the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (impaired pathways in cancer). In cancer cells, TCA intermediates are exported from the mitochondrial matrix and become available for the synthesis of fatty acids. [$1\text{-}^{13}\text{C}$]glucose fluxes through glycolysis and the TCA cycles can be followed using ^{13}C -NMR (FIGURE 2).

The use of quantitative and/or flux-based metabolic approaches in conjunction with classic biochemical assays has demonstrated that in cancer, metabolic changes are more commonly related to altered kinetic activities of enzymes. Increased lactate production was accompanied by increased activities of hexokinase 1 and 2, phosphofructokinase, aldolase, M2-pyruvate kinase and LDH, all key enzymes of glycolysis (FIGURE 2) [27–31]. In addition, ATP citrate lyase and fatty acid synthase, which synthesize long-chain fatty acids from citrate and acetyl-CoA, are upregulated or activated in many cancers [27,28,30]. In addition to the upregulation of glycolytic enzymes, downregulation of the TCA-cycle enzymes and oxidative phosphorylation has been reported. Pyruvate dehydrogenase (PDH, one of the key enzymes in the TCA cycle) activity is inhibited through phosphorylation by pyruvate dehydrogenase kinase (PDK) 1, which being a hypoxia inducible factor (HIF)-1 target gene, is highly upregulated in various cancers [27]. Mitochondrial DNA abnormalities, mostly due to a combined complex I/III defect and increased reactive oxygen species production, have been reported for a variety of cancers [32,33].

Cell proliferation, vitality and biosynthesis rely highly on energy state. Therefore, the most obvious explanation for increased glycolysis is the rapid (but not efficient) production of ATP, especially in hypoxic cancers. The net result of lactate production is two ATP molecules, while the mitochondrial pathway yields 36 ATP molecules from the one glucose molecule metabolized (FIGURE 2) [34]. Therefore, more glucose molecules need to be utilized in order to produce enough ATP through rapid but inefficient glycolysis. Recent evidence demonstrates, however, that the increased energy demand is only a partial explanation for the increased glycolytic activity, especially in metastases. Lactic acid is the principal end product of glycolysis and is exported from the cancer cell through the monocarboxylate transporter-4. Once exported, it results in an acidic tumor microenvironment that favors tumor invasion [28,35,36].

One hypothesis for the possible molecular mechanisms leading to the upregulation of glycolysis in cancer cells is related to the HIF-1 pathway. It has been demonstrated that stabilization of the HIF-1 transcription factor leads to upregulation of LDH transcription and other genes involved in glucose transport, metabolism and pH regulation [37]. HIF-1 also induces PDK1 transcription, which inactivates PDH (as mentioned above) and as such inhibits the major key enzymes of the mitochondrial TCA cycle. However, in 2004, Craig Thompson reported that activated AKT (upstream regulator of the HIF-1 signaling pathway), independent of HIF-1, can convert cancer cells to glycolysis [38]. Since then, pioneering work has provided evidence that upregulation of the AKT/PI3K/ TOR signal transduction pathway (upstream of HIF-1 stabilization) induces hexokinase II activity, which by the attachment to mitochondrial porin, redirects mitochondrial ATP to phosphorylate glucose and drives glycolysis (FIGURE 3) [33]. Interestingly, it has recently been shown that it is not only AKT that stimulates glycolysis, but that defects in mitochondrial respiration can lead to activation of AKT [29,

30] , revealing a complex pathway of crossregulation between signal transduction pathways and metabolic programming in cancer cells.

Concurrent with these basic research discoveries on the enhanced glycolytic phenotype of cancer cells has been the increasing clinical use of FDG-PET, which is based on the observation that glucose uptake is functionally altered in a wide variety of human cancers [27,39] .

Kennedy pathway & phospholipid metabolism

Phosphatidylcholine (PC) is the major phospholipid in the cell membrane. In cancer, choline metabolism is characterized by an elevation of phosphocholine (PCho) and total choline-containing compounds [17,40] . Recent evidence demonstrates that increased PCho concentration is not a simple indicator of the increased phospholipids production necessary for cancer cells to meet higher proliferation requirements, but likely represents the result of activation of PC-cycle enzymes, caused by genetically induced changes in growth factor-mediated cell signaling pathways. Genetic alterations, as well as changes in Ras signaling pathways, affect expression and post-translationally regulated activity levels of these enzymes, thereby leading to changes in the overall choline phospholipid metabolite profile [17] . The *de novo* biosynthesis of PC occurs via the three-step Kennedy pathway in which choline is phosphorylated into PCho by choline kinase (Chok), PCho is converted into cytidine 5-diphosphocholine (CDP)-choline by cytidylyltransferase and CDP-choline is incorporated into PC by choline phosphotransferase (FIGURE 4) [17,40–42] .

Chok activity, mRNA, and protein levels were elevated in a rat colon-cancer model and Chok activity and PC levels were increased in human colon cancers and adenomas [43]. Overall, phosphorylation of choline to PCho is increased five- to 12-fold in various cancers [44]. Also, choline transport into the cell has been demonstrated to be increased in malignant cells owing to overexpression of the choline high-affinity transporter-1 and choline transport-like protein, which are highly expressed in the colorectal cancer cell line, HT-29 [44–46]. In recent years, it has been demonstrated that in patients with early-stage non-small-cell lung cancer, expression of Chok is prognostic: 4-year lung cancer-specific survival was lower for patients with elevated Chok expression compared with patients with low levels of the enzyme (46.66 vs 67.01%, respectively) [47] . Downregulation of Chok by using siRNAs against Chok was associated with decreased proliferation, increased differentiation and increased sensitivity to 5-fluorouracil in breast cancer cell lines [48,49] , suggesting possible therapeutic targets of the Kennedy pathway.

Similarly to glucose metabolism, HIF-1 signaling has also been shown to be involved in upregulation of the Kennedy pathway [50] . HIF-1 can directly bind to the endogenous Chok promoter, and trigger upregulation of Chok and choline phosphorylation. Again, another HIF-1-independent activation of the Ras/Raf/ ERK/MAPK pathway in human breast as well as colorectal cancer cells, was associated with increased levels of PCho [51] . Inhibition by a MEK inhibitor, U0126, was associated with a time-dependent decline in PC levels, which correlated well with the decrease in ERK1/2 phosphorylation [52] (FIGURE 4). The same group has also demonstrated that blockade of PI3K with LY294002 (as evidenced by decreased P-AKT levels) was associated with a partial fall in PC level [53] . However, the precise molecular mechanisms of Kennedy pathway regulation by RAS/ Raf/ERK and/or by PI3K/AKT pathway (to a lesser extent), remain unclear.

Similar to FDG-PET protocols for glucose uptake, clinical protocols are under development in order to monitor choline uptake and metabolism in cancer patients using ¹H-MRS/MRSI and fluorine-18- or carbon-11-labeled choline derivatives by PET [17,20,54] .

Novel anticancer agents

Cytotoxic chemotherapeutics are a major component of the treatment of neoplastic disease. Chemotherapeutic agents are used for the initial treatment of several types of solid tumors, as well as in the neoadjuvant and adjuvant setting and in palliative care. Cytotoxic agents are capable of reducing tumor burden, prolonging life and even cure. The primary mechanism of action of chemotherapeutic agents is perturbation of the cellular processes involved in proliferation: DNA synthesis and cell division. These effects often result in cell death by either apoptosis or necrosis. There are several different classes of chemotherapeutic agents; alkylating agents, antimetabolites and natural products. Here, we provide a cursory overview of the different classes of agents and their general mechanisms of action. More detailed reviews may be found elsewhere [55,56].

Alkylating agents were among the first agents to be used for systemic cancer therapy and include groups of agents such as nitrogen mustards (cyclophosphamide, melphalan, mechlorethamine), nitrosoureas (carmustine, streptozocin), triazenes (dacarbazine, temozolomide) and platinum analogs (cisplatin, carboplatin, oxaliplatin). Because alkylating agents bind directly to DNA and cause either single-strand breaks or crosslinking of DNA, their activity is not cell-cycle specific and therapeutic efficacy is primarily dependent upon exposure (area under curve), and is relatively independent of schedule. Antimetabolites consist of folic acid analogs (methotrexate, pemetrexed), pyrimidine analogs (5-fluorouracil, cytosine arabinoside, gemcitabine), and purine analogs (6-mercaptopurine). Antimetabolites disrupt DNA synthesis and cell division by the inhibiting formation of normal nucleotides or by direct interaction with DNA, which prevents extension of DNA strands. Most antimetabolites exert their effects in a specific phase of the cell-cycle and, as a result, the drugs can only have an effect on a fraction of the dividing cells at any given time. Therefore, the duration of exposure (the time above a critical threshold concentration) is more important than achieving maximum plasma drug concentrations. Natural products consist of several different types of agents that elicit their effects in a variety of ways. Vinca alkaloids (vinorelbine and vinblastine) and taxanes (paclitaxel and docetaxel), are antimitotic agents that disrupt the microtubule polymerization and depolymerization processes. Camptothecin analogs (topotecan and irinotecan) bind to and stabilize the topoisomerase I–DNA complex, which ultimately results in an irreversible double-strand DNA break. Both the antimicrotubule agents and camptothecin analogs are cell-cycle specific and toxicity is dependent upon both drug concentration and duration of exposure. There are several mechanisms that contribute to anthracycline antibiotic (daunorubicin, doxorubicin, epirubicin, idarubicin and mitoxantrone) cytotoxic effects; DNA intercalation, which directly affects transcription and replication, interaction with the DNA–topoisomerase II complex, which inhibits the relegation of DNA strands, and the generation of free radicals, which can oxidize DNA nucleotide bases.

Chemotherapeutics are primarily administered on a maximum tolerated-dose (MTD) schedule [57]. The premise behind MTD dosing is that, in general, larger fractions of the tumor cell population are killed at higher concentrations of cytotoxic agents. Therefore, the idea is to expose the tumor to the highest possible dose as frequently as possible before serious toxicity occurs. Implementation of MTD-based dosing is further justified by the proven dose–response effect of tumors to several different cytotoxic chemotherapeutic agents, including cyclophosphamide, doxorubicin, methotrexate and cisplatin [58,59]. However, since chemotherapeutic drugs nonspecifically target dividing cells, some normal dividing cells are affected, causing side effects such as neutropenia, myelosuppression, alopecia, intestinal mucosa damage-induced diarrhea, nausea, mucositis, and neurological and reproductive damage. The harsh side effects necessitate a recovery or ‘break’ period that usually spans 2–3 weeks. Breaks in conjunction with growth-factor support, frequently used to help accelerate recovery from toxicity [60], allow the tumor to repopulate and damaged tumor vasculature to

repair [61]. Similar to the dividing tumor cells, proliferating endothelial cells of the tumor vasculature should be susceptible to conventional chemotherapeutic drugs. However, antiangiogenic effects are not optimized when chemotherapeutics are given on a MTD schedule [57].

The administration of chemotherapeutics at lower doses at more frequent intervals, termed 'antiangiogenic' or 'metronomic' scheduling, has been shown to reduce host toxicity. Metronomic chemotherapeutic scheduling has proven to be effective in both preclinical models [57,62] and in the clinic [63]. Docetaxel [64], paclitaxel [65], vinblastine [66] and cyclophosphamide [57] are some of the cytotoxic agents that have demonstrated antiproliferative activity on endothelial cells in culture.

Progressive disease, the process of tumor growth, angiogenesis, invasion and metastasis, is largely regulated by growth-factor signaling and their binding to receptor tyrosine kinases [67]. Inhibition of these signaling pathways as a therapeutic approach has recently gained a lot of attention and current strategies include: anti-growth factor antibodies, receptor antagonism, antireceptor monoclonal antibodies, antisense and small-molecule TKIs [68,69]. The use of molecularly targeted anticancer drugs began with the introduction of Gleevec® (Novartis, Basel, Switzerland) which targets BCR-ABL and Kit, for the treatment of chronic myeloid leukemia [70]. Some of the signal transduction pathways commonly altered in the malignant phenotype include: VEGF, VEGF receptor 2 (KDR/Flk-1), EGFR (erbB1) and erbB2; as well as downstream signaling kinases MEK, Raf and AKT [68,69].

It has been demonstrated that the antiproliferative effects of cytotoxic chemotherapy can be abrogated by VEGF and other growth factors [64,71]. Therefore, it is rational to hypothesize that the addition of molecularly targeted inhibitors to chemotherapeutic regimens may improve therapy. Indeed, several clinical studies have now demonstrated that incorporation of molecularly targeted agents with existing therapies, radiation therapy and/or chemotherapy, result in improved efficacy with no increase in toxicity [72–74].

Although several molecular agents have demonstrated promising single-agent efficacy in pre-clinical models, the use of molecularly targeted agents in the clinic will almost certainly involve combinations with other therapeutic modalities. This discrepancy is partly owing to resistance development to target agents due to molecular mutations at the binding site of the target receptor. Since treatment with molecular agents results primarily in delayed tumor progression and not necessarily tumor regression, monitoring therapeutic efficacy and assessing the potential clinical utility of new agents (i.e., in early Phase I and II trials) poses a difficulty.

Metabolic signatures of resistance

The use of metabolomics for assessment of treatment effect, as both a predictive measure of efficacy and as a pharmacodynamic marker, has been demonstrated *in vitro* for both traditional chemotherapy and hormonal agents. Use of ¹H-NMR on human glioma cell culture successfully predicted separation into drug-resistant and drug-sensitive groups prior to treatment with 1-(2-chloroethyl)-3-cyclohexyl-1-nitro-sourea [75]. Exposure of hormone-responsive Ishikawa human endometrial adenocarcinoma cells to tamoxifen resulted in dose-dependent changes in nucleotides, suggesting that tamoxifen modifies RNA translation [76].

In vivo, ¹H-NMR, including HR-MAS, has been used to investigate the metabolic changes associated with nitrosourea treatment of B16 melanoma and 3LL lung carcinoma tumors grown subcutaneously in C57BL/6J mice [19]. During the growth-inhibitory phase, tumor samples demonstrated significant accumulation of glucose, glutamine, aspartate and serine-derived metabolites, as well as decreased succinate, suggesting the reduction of nucleotide synthesis

and induction of DNA repair pathways. Growth recovery reflected metabolic adaptation, including activation of energy production systems and increased nucleotide synthesis.

In prostate cancer, citrate may be a marker of responsiveness to treatment based upon a pilot study where 16 high-risk prostate cancer patients were treated with chemotherapy, hormones, radical prostatectomy or radiotherapy, and subsequently followed with prostate-specific antigen monitoring, MRI and MRSI [77,78]. A risk score utilizing MRSI was developed based on both tumor volume and metabolic abnormality. The MRSI score and MRI tumor/node stage was then used to determine prostate-specific antigen relapse and was predictive in 15 of 16 cases.

There is an ongoing effort by the National Cancer Institute, researchers, clinicians and industry to expand the use of metabolomics, with particular attention to MRSI for the assessment of therapeutic response [79]. In general, a decrease in the Cho signal on ^1H -NMR equates to a response to chemotherapy or radiation, and may be an early marker of effect as it can be detected prior to changes on conventional imaging in breast and prostate cancer, brain tumors, and non-Hodgkin's lymphoma.

As mentioned above, the practice of oncology has evolved from the exclusive use of cytotoxic compounds that nonselectively inhibit cells actively engaged in the cell cycle to include newer targeted agents that can block particular pathways important for neoplastic transformation, growth and metastasis. Without being truly cytotoxic (no immediate cell death) but rather cytostatic, it is increasingly important to apply sensitive quantitative imaging end points to monitor therapy response. Preclinical testing is a critical component of the development of new therapeutics and new therapeutic strategies. With increasing interest in molecularly targeted therapies, where dosing to toxicity may not provide the greatest benefit, and the use of alternative dosing schedules for cyto-toxic chemotherapeutics, the establishment of clear, quantitative and robust preclinical trans-lational end points of therapeutic efficacy is necessary [80].

Therapeutics in oncology is moving toward the use of drugs that specifically target aberrant pathways involved in growth, proliferation, and metastases. Biomarkers are being increasingly utilized in the early clinical development of such agents to identify, validate and optimize therapeutic targets and agents, determine and confirm mechanism of drug action, as a pharmacodynamic end point, and in predicting or monitoring responsiveness to treatment, toxicity and resistance [81]. Metabolomics has a potential future role as a biomarker and current examples of its use in developmental therapeutics include TKIs, proapoptotic agents, and heat shock protein inhibitors [6,18,82–86].

One hypothesis explored was that treatment with targeted therapies, such as signal transduction inhibitors, would result in a distinct metabolic profile between sensitive and resistant cells. Imatinib, a TKI of the *BCR-ABL* oncogene, decreases cell proliferation and induces apoptosis in human chronic myeloid leukemia [6,18]. Metabolically, imatinib interrupts the synthesis of macromolecules required for cell survival by deprivation of key substrates. Investigation of glucose metabolism changes in imatinib-treated human leukemia *BCR-ABL*-positive cell lines with NMR demonstrated decreased glucose uptake by glycolysis inhibition, but unlike classic therapeutics it stimulated mitochondrial metabolism, leading to cell differentiation [18]. Imatinib also led to a significant decrease in PC in imatinib-sensitive cells, which correlated with a decrease in cell proliferation rate [18]. Metabolomic detection of imatinib resistance has also been reported; a decrease in mitochondrial glucose oxidation and nonoxidative ribose synthesis from glucose, as well as highly elevated phosphocholine levels, was indicative of drug resistance and disease progression [6]. These data indicate that NMR metabolomics may provide a method for monitoring changes in cellular metabolism that reflect

early resistance to novel targeted agents (FIGURE 5). This could be particularly useful in hemato-logical malignancies, where frequent tissue sampling is feasible and early metabolomic markers of resistance may dictate therapy adjustments that prevent overt phenotypic progression.

Apoptosis has an established role in chemotherapy and radiation-induced cell death and its absence correlates with treatment resistance and induction of pro-survival pathways [59,60]. Multiple new agents targeting apoptosis are currently in early clinical development including TNF-related apoptosis-inducing ligand (TRAIL), agonist death receptor antibodies, and inhibitors of the antiapoptotic proteins. FK866 is a novel agent that purportedly induces apoptosis independent of anti-DNA effects by decreasing NAD^+ levels [82]. The metabolic effects of FK866 on mouse mammary carcinoma cells using ^1H -NMR demonstrated a significant increase in fructose-bi-phosphate with a subsequent decrease in pH and NAD^+ resulting from an incomplete glycolytic cycle [82]. Other alterations observed included changes in guanylate synthesis, pyridine nucleotide levels and phospholipid metabolism, indicating multiple aberrant cellular pathways resulting in apoptosis [83]. High-resolution MAS-NMR has demonstrated that apoptotic activity can be characterized in cervical carcinoma biopsies before and during treatment with external beam radiation, brachytherapy and weekly chemotherapy [84]. In this study, 44 cervical cancer biopsies indicated that lipid metabolism differed both in tumor cell fraction (percentage of tumor cells per tissue biopsy) and tumor cell density (number of carcinoma cell nuclei per mm^2 of tumor tissue). Ratios of fatty acid $\text{CH}_2:\text{CH}_3$, specifically, a lengthening of the fatty acid- CH_2 chain, were associated with apoptosis, a discovery substantiated in another study of acute lymphoblastic leukemia cell cultures treated with doxorubicin [85]. These early studies suggest metabolomics may be a useful biomarker in the development and validation of proapoptotic agents.

Another interesting application of metabolomics is in the area of heat-shock protein 90 (Hsp90) inhibitors. Although their mechanism of action is not fully elucidated, current data suggest that this family of agents increase the cellular destruction of client oncogenic proteins. In one study, colon cancer xenografts were treated with an Hsp90 inhibitor and extracts of these tumors were analyzed by ^{31}P -NMR, reflecting a significant increase in phosphocholine, phospho-monoester/phosphodiester ratio, valine and phosphoethanolamine levels, indicating altered phospholipid metabolism [86]. These results, although preliminary, address metabolic changes that could be used as pharmacodynamic biomarkers of Hsp90 inhibitors, a class of agents that do not appear to result in classical anti-tumor effects.

A summary of metabolic changes observed upon treatment with cytotoxic and cytostatic agents is presented in TABLE 3. If resistance occurs, these metabolic markers cannot be detected in the cancer cells: increased glucose, lactate and PCho are observed.

***In vivo* metabolic imaging for response assessment**

The dominant modality for imaging tumor biochemistry *in vivo* is PET [87–89]. The majority of clinical PET studies are based on intravenous injection of ^{18}F FDG, which is a marker for hexokinase activity (the rate-limiting step in glucose metabolism). Because most malignancies exhibit increased glucose uptake and glycolytic metabolism, as mentioned above, whole-body ^{18}F FDG-PET can provide information on tumor aggressiveness and is now recognized as a procedure of choice for the purpose of staging, restaging and monitoring response to treatment of various types of cancers. Glucose dependence in various malignancies relies on overexpression of glucose transporters (mainly Glut-1), and key enzymes of glycolytic pathways, such as hexokinase, pyruvate kinase and phosphofructokinase. Hence, FDG is transported into the cell by the energy-independent Glut-1 transporter. Unlike natural glucose, however, FDG is trapped inside the cell because it cannot be metabolized by glucose-6-

phosphate isomerase. Overall, FDG uptake appears to be a marker of high-grade and/or poorly differentiated tumors [89]. In addition, there is a strong correlation between the size of the tumor and FDG accumulation as a tumor progresses. Today, metabolic FDG-PET imaging in combination with anatomical CT is routinely used for the evaluation of solitary pulmonary nodules, for the initial staging of newly diagnosed malignancies, for the detection of clinically or radiologically suspected recurrences, and in the evaluation of tumor response during the course of treatment [90–94]. It has recently been demonstrated that for novel targeted STMs, decreases in metabolic aggressiveness and the glycolytic pheno-type precede changes in anatomic size. As such, early detection of therapeutic responses to chemotherapy, mTOR inhibitors, EGFR TKIs, CKIT inhibitors and, most recently, IGF-1R antibodies [89–94] have been detected by FDG-PET in various tumors. Decrease in FDG uptake as an assessment of therapy response has been seen in patients with head and neck carcinoma, as well as lymphoma, breast, lung and colorectal cancers. For the majority of tumors (such as head and neck, non-small cell lung cancer and cervical cancer), metabolic PET was superior to CT in radiotherapy planning and assessment of therapy response. Several studies have indicated that in responding tumors, FDG uptake markedly decreases within the first chemotherapy cycle or after 14 days of treatment [95,96]. In gastric cancer patients treated with chemotherapeutics, metabolic responders by PET also demonstrated a high histopathologic response rate (69%) and favorable prognosis, whereas metabolic non responders have a poor prognosis and only 17% demonstrated histopathologic response [97]. Metabolic response to targeted drugs, such as the c-KIT-targeted TKI imatinib in gastrointestinal stromal tumor patients, can occur in the first hours after treatment begins and can be used to adjust the patient's daily dose [98]. Even in hepatocellular carcinoma patients, known to have low FDG uptake on liver PET scans at baseline, after 3 weeks of sorafenib treatment (a multi-targeted VEGF receptor 2 TKI) a partial metabolic response was observed in responders versus non-responders [99]. Preclinically, glucose metabolic activity closely reflected response to the EGFR TKI gefitinib [100]. In addition, novel radiotracers, related to various metabolic abnormalities in cancer metabolism, are currently under development. For example, the use of technically improved PET/CT scanners with new ^{11}C and ^{18}F choline tracers are under development.

Metabolic PET imaging obviously requires exogenous radioactive tracers. By contrast, *in vivo* MRS, in particular ^1H MRS, can simultaneously provide several molecular images using endogenous metabolites [101]. In the breast, increased choline signal (due to increased cell membrane phospholipid synthesis) can be seen on ^1H -MRS (or MRSI) scans, which correlates well with anatomical localization of the tumor lesion and response to the therapy [17,101]. Thus, abundant metabolic markers, distinguished by *in vitro* NMR-based metabolomics, can be translated into clinical '*in vivo* omics'-based imaging by PET and MRS.

Conclusion

Although there have been major advances in the treatment of human cancer in the last 10 years, it remains a life-threatening disease for which new treatment strategies are urgently needed. This is especially true for patients whose disease recurs and progresses following treatment. Cancer development is a time-dependent accumulation of several events, both genetic and epigenetic, which will result in changes in genes, proteins and endogenous metabolites. New molecularly designed drugs, which target specific abnormalities in the cancer cell (such as onco-tyrosine kinase, antibodies to growth factors) and act cytostatically, will lead to normalization of metabolic cell phenotype in responsive patients, even though the specific metabolic markers can vary depending on the targeted pathway and/or cancer phenotype. These metabolic changes, as well as establishing a drug-resistant phenotype, can be assessed using global metabolic profiling followed by identification and validation of a specific metabolic marker(s). Ideally, selected metabolic biomarkers can then be followed non-invasively using 'molecular imaging' – a novel term in the clinical setting since 2000 – referring to *in vivo*

visualization of endogenous genes, proteins and metabolites noninvasively in the body using external molecular probes and advanced radiological techniques such as MRSI and/or novel metabolic tracers for MRI and PET.

In addition to its potential for cancer detection and in assessing the efficacy of anticancer drugs, the metabolic findings, gained through metabolomics technology, can be applied to combat cancer directly. Glucose metabolism and choline turnover represent two major upregulated metabolic pathways in cancer. Therefore, compounds that limit glycolysis would, in theory, kill cancer cells while sparing normal cells that can burn amino and fatty acids for energy through mitochondrial pathways. The enzymes directly causing the elevation of phosphocholine and total choline-containing compounds, such as choline kinase, phospholipase C and D, may also provide molecular targets for anticancer therapies.

In summary, metabolomics has the potential to influence clinical oncology, benefiting patient care with advantages already being seen in the use of metabolite imaging in breast and prostate cancer diagnosis and probable future uses as a biomarker for early cancer diagnosis, treatment efficacy and developmental therapeutics. Metabolomics, when used as a translational research tool, can provide a link between the laboratory and clinic, since metabolic and molecular imaging enables the discrimination of metabolic markers noninvasively *in vivo*. Of all possible applications for NMR, cancer research is the most advanced field already utilizing absolute biochemical values rather than pure spectral analysis. Precise biomarker validation and quantification in *ex vivo* samples and their translation into *in vivo* metabolic imaging will ultimately guide future therapeutic strategies with novel targeted anticancer drugs.

Future perspective

The future of imaging promises new avenues for exploration of molecular, microscopic, genetic and biologic events in humans during oncologic development and/or response to anticancer treatment, performed noninvasively and in real time. Accordingly, new quantitative multimodality protocols with improved accuracy, specificity and sensitivity can be applied in different phases of cancer development – from detection to diagnosis, staging and therapeutic response – from animal model into clinical practice. Introduction of metabolic inhibitors, targeting glycolytic and Kennedy pathway enzymes, possess significant clinical therapeutic potential. Improvements in hardware technology and image processing will allow the routine acquisition of high-quality, high-resolution, multiplanar images of the whole body by CT and MRI. Development of novel metabolic and molecular radioactive probes will allow for future establishment of PET technology as a reliable tool for clinical molecular imaging.

Executive summary

- Biomarkers are increasingly being utilized in the early clinical development of novel anticancer drugs to identify, validate and optimize therapeutic targets and agents, determine and confirm mechanism of drug action, as a pharmacodynamic end point, and in predicting or monitoring responsiveness to treatment, toxicity and resistance.
- The major advantage of nuclear magnetic resonance-based metabolomics is its translational components, which range from a pattern-recognition technique to precise metabolite identification, validation and finally, noninvasive assessment by magnetic resonance spectroscopy, magnetic resonance spectroscopic imaging or, most recently, PET.
- Glucose metabolism (Warburg effect) and choline turnover (Kennedy pathways) are two major pathways upregulated in cancer.

These pathways are also sensitive markers for therapy response (decreased glucose uptake, decreased glycolysis and decreased choline phosphorylation).

- In resistant tumors, these two pathways remain upregulated.
- An accurate assessment of metabolic phenotype and fluxes of cancer cells is essential to design effective metabolic modulators to help to overcome resistance development to various targeted agents.
- Similar to genomics and proteomics, metabolic pattern recognition approaches, based on global metabolic profiling, are challenged due to lack of a standard technology platform (especially for statistical postprocessing analysis) and lack of validation of comprehensive data bases, often accompanied by poor study design.
- Precise marker identification, validation and translation into noninvasive metabolic imaging protocols are required in metabolomics in order to become an established clinical tool.

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Figure 1. Potential applications and benefits of genomics, proteomics and metabolomics technologies in individualized medicine for cancer patients

Conventional genomics (global gene or DNA mapping) and functional genomics (transcriptomics or RNA mapping) has been used mostly on biopsy samples for early assessment of tumor predisposition and classification. The routine analysis of large endogenous molecules such as proteins and peptides, both in tissues and body fluids, became feasible with the advances in MS and can be used for early diagnosis and patient selection for targeted therapies. NMR spectroscopy and MS are metabolomics techniques that allow for the simultaneous assessment of hundreds of low-molecular weight endogenous metabolites as well as their dynamic fluxes. Identified metabolic markers can then be translated into *in vivo* metabolic magnetic resonance spectroscopy/MRSI and PET imaging protocols for therapeutic monitoring.

IEF: Isoelectric focusing; MRSI: Magnetic resonance spectroscopical imaging; MS: Mass spectroscopy; NMR: Nuclear magnetic resonance; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Figure 2. [1-¹³C]glucose metabolic pathway

In cancer cells, increased uptake of [1-¹³C]glucose through the glucose transporter Glut-1 is detected by analyzing extracellular levels of [1-¹³C]glucose in media. Increased activity of glycolysis is calculated by the amount of intracellular [3-¹³C]lactate in the cellular fraction, as well as the amount of lactate exported into media through the MCT4. Decreased mitochondrial activity is detected by low levels of ¹³C-enrichment into [4-¹³C]glutamate (through pyruvate dehydrogenase) and [2,3-¹³C]glutamate (through pyruvate carboxylase). Finally, exported acetyl-CoA (from the truncated tricarboxylic acid cycle) into the cytosol is used for increased ¹³C-fatty-acid synthesis. ¹³C-fluxes through the pentose phosphate pathway can be detected by alternative methods, such as gas or liquid chromatography.

3P: Tri-phosphate; ACL: ATP citrate lyase; FAS: Fatty acid synthase; LDH: Lactate dehydrogenase; M2-PK: M2 pyruvate kinase; MCT: Monocarboxylate transporter; OXPHOS: Oxidative phosphorylation; PFK: Phosphofructokinase.
Adapted from [6].

Figure 3. Metabolic reprogramming by PI3K/AKT/TOR and Ras/Raf/ERK/MAPK signal transduction pathways

Two major oncogenic pathways, the GTPase Ras/Raf/ERK/MAPK and the lipid kinase PI3K/AKT/mTOR pathways, are downstream signaling pathways for various oncogenic surface tyrosine kinases, such as IGF-1R or EGF receptor. The AKT signaling pathway has been shown to upregulate glucose metabolism (increased activity of Glut-1 and glycolytic enzymes, as well as inhibition of pyruvate dehydrogenase by overexpressing of PDK). The Ras pathway is involved in overexpression of Chok in the Kennedy pathway.

Cho: Choline; Chok: Choline kinase; IGF-1R: IGF receptor 1; IRS: Insulin receptor substrate; LDH: Lactate dehydrogenase; PDK: Pyruvate dehydrogenase kinase; PFK: Phosphofructokinase.

Figure 4. Biosynthetic (solid lines) and catabolic (dashed lines) pathways of choline phospholipid metabolism (the Kennedy pathway)

Choline is an essential nutrient and is transported into the cell by choline transporters. Choline transporter-like protein is the major choline transporter in human cancer cells.

Phosphatidylcholine is the major membrane phospholipid. Choline kinase, which phosphorylates choline into phosphocholine, acts as a regulatory enzyme for phosphatidylcholine synthesis. Overexpression and increased activity of choline transporters and choline kinase has been demonstrated in malignant cells, resulting in increased levels of phosphocholine and phosphatidylcholine.

CDP: Cytidine 5-diphosphocholine; CDP-choline: Citicholine; CMP: Cytidine mono-phosphocholine; CTP: Cytidine choline transferase; PPi: Pyrophosphate.

Adapted from [17].

Figure 5. PLS-DA ana lysis and marker validation to distinguish metabolic signature of responsiveness and resistance to imatinib in human Bcr-Abl-positive cells from CML patients
The leukemic cells, sensitive and resistant to the Bcr-Abl tyrosine kinase inhibitor imatinib, were treated with 1 μM imatinib for 24 h. Statistical PLS-discriminant ana lysis on high-resolution ^1H -NMR spectra (both extracts and medium spectra sets were used) allows for group clustering: sensitive untreated cells (squares) versus sensitive cells treated with imatinib (circles) versus resistant cells treated with imatinib (triangles). The group clustering was based on changes in glucose, lactate, choline intermediates and glutamine, with minor contribution of creatine and alanine (see 2). For three steps in metabolomics ana lysis (pattern recognition:metabolite identification:biomarker validation) refer to (1).
Cho: Choline; CML: Chronic myeloid leukemia; GPC: Glycerophosphocholine; PC: Phosphatidylcholine; PLS-DA: Partial least square discriminant analysis.

Table 1

Summary of biomaterials used for ^1H -NMR metabolic analysis in various areas of biomedical research.

Biofluid	Required sample handling
Urine	Add deuterated phosphate buffer to 0.5–3 ml urine
Blood	For 0.5 ml of heparinized blood product:
Plasma	- Only deuterium oxide addition (lock)
Serum	- Acetonitrile addition (protein precipitation)
	- Methanol/chloroform extraction (lipid separation)
CSF	Add deuterium oxide to 0.5 ml CSF
EPS	Add deuterium oxide to 0.03–0.10 ml EPS
Bile	Add deuterated methanol to 0.5 ml bile
BALF	Add deuterium oxide to 0.5 ml BALF
Tissue	- Add 0.01 ml deuterium oxide to 3–10 g tissue in MAS rotor - Perchloric acid extraction on 20–200 mg frozen tissue - Methanol/chloroform extraction on 20–200 mg frozen tissue

BALF: Bronchoalveolar lavage fluid; CSF: Cerebrospinal fluid; EPS: Expressed prostatic secretion;

Table 2

Metabolic phenotype of human tumors assessed by magnetic resonance technique.

Metabolic marker	Biochemical mechanisms	Assay	Ref.
ATP (general) ↓	Decreased ATP production due to glycolytic phenotype; increased ATP utilization due to fast cell proliferation rate	³¹ P-NMR	[3,13,18,32,34,79]
Citrate(prostate cancer) ↓	Decreased in prostate tumors due to disturbances in Zn metabolism	¹ H- and ¹³ C-NMR MRSI, MRS	[3,12,21,78]
Extracellular pH (general) ↓	Acidic microenvironment in tumors due to increased production of lactic acid	³¹ P-NMR	[3,36]
Glucose uptake (general) ↑	Increased Glut transporter activity due to glycolysis-derived ATP production	¹³ C-NMR, PET	[2,3,6,18,22,26–39]
Glycerophosphocholine (general) ↓	Catabolic product of membrane phospholipids; decreased due to downregulated apoptosis (for majority of cancers, excluding prostate cancer)	¹ H- and ³¹ P-NMR	[2,3,17,18,40–44]
Myo-inositol (glioma) ↑	Increased proliferation of astroglial cells that express a high amount of the osmoregulator myo-inositol	¹ H-NMR and MRS	[2,3,13,16]
Lactate (general) ↑	Increased activity of glycolytic enzymes in presence of oxygen (the Warburg effect)	¹³ C- and ¹ H-NMR	[2,3,18,27–39]
NAA (brain) ↓	Decreased neuronal functions with high-grade gliomas, astrocytomas and neuroblastomas lead to decreased levels of the osmolyte NAA	¹ H-NMR and MRS	[2,3,13,16]
NAD ⁺ (general) ↓	Decreased mitochondrial metabolism (decreased TCA cycle and respiration)	³¹ P-NMR	[2,3,32–34,83]
Phosphocholine (general) ↑	Precursor for membrane phospholipids; enzyme induction owing to high proliferation rate	¹ H- and ³¹ P-NMR, MRSI, MRS and PET	[22,3,17,18,40–53]

¹³C-NMR: Carbon NMR using hyperpolarized gases; MRS: Magnetic resonance spectroscopy; MRSI: Magnetic resonance spectroscopic imaging; NAA: N-acetyl aspartate; NAD⁺: Nicotinamide adenine dinucleotide; NMR: Nuclear magnetic resonance; TCA: Tricarboxylic acid; Zn: Zinc.

Table 3

Metabolic consequences of anti-cancer treatment.

Anticancer drug	Mechanism of action	Tumor site	Metabolic markers & consequences	Ref.
Cisplatin	Cytotoxic	Melanoma	ATP↓ , glucose↓	[2,4,94,96]
Cystemustine	Cytostatic	Melanoma	All phospholipids↑, glucose↑, glutamine↑, PUFA↑, lipidst	[11,19]
Dexamethasone	Cytostatic	Leukemia	Phospholipids↓ (sensitive) Phospholipids↑ (resistant)	[4,17]
5-fluorouracil	Cytostatic	Breast	Mobile lipids↑, total choline↑, PC↓	[1717,23,94]
FK866	Apoptosis	Breast	Fructose-bi-phosphate↑, PUFA↑, NAD+↓	[82]
Ganciclovir	Gene therapy	Glioma	PUFA↑, myo-inositol↓ , taurine↓ , glycine↓	[86]
Imatinib	Bcr-Abl TKI	Leukemia	Glc↓ , lactate↓ , TCA cycle↑, ATP↑, PC, GPC↑	[6,17,18,93,98]
Indomethacin	COX2 inhibitor	Breast	PC↓ , GPC↑ at 2 h	[17]
LY294002	PI3K TKI	Breast	PC ↓ up to twofold GPC↑ up to fivefold	[53]
Methotrexate	Cytotoxic	Glioma Breast	[Cho/water]↓ No changes	[17]
MN58b	Choline kinase inhibitor	Breast Colon	PC↓ , choline kinase↓	[102]
Radiation	Cytotoxic	Various	Total Cho↓ , mobile lipidst, glucose↓	[17,94,101]
Resveratrol	Cytotoxic	Leukemia	ATP↓	[4]
Semaxanib	VEGFR, KIT-TKI	Sarcomas	¹⁸ FDG uptake↓	[4,74,97]
Tamoxifen	Cytotoxic	Glioma	[Cho/Cr]↓ , [Lac/Cr]↓ , [NAA/Cho]↑	[17]
Temozolomide	Cytotoxic	Glioma	[Cho/water] ↓	[17]
U0126	MAPK inhibitor	Breast Colon	PC ↓ to 74% at 16 h and to 23% at 48 h	[52]

Cho: Choline; c-KIT: CD117 cytokine receptor; COX: Cyclooxygenase; Cr: Creatine; FDG: Fluoro-deoxy-glucose; GPC: Glycerophosphocholine; Lac: Lactate; MAPK: Mitogen-activated protein kinase; NAA: N-acetyl aspartate; PC: Phosphocholine; PI3K: Phosphoinositide 3-kinase; PUFA: Poly-unsaturated fatty acid; TCA: Tricarboxylic acid; TKI: Tyrosine kinase inhibitor; VEGFR: VEGF receptor.